

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 15/19, C07K 14/52, 16/24, A61K 38/19		A1	(11) International Publication Number: WO 95/18858 (43) International Publication Date: 13 July 1995 (13.07.95)																																																								
(21) International Application Number: PCT/US94/14553		(71) Applicant (for all designated States except US): GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).																																																									
(22) International Filing Date: 28 December 1994 (28.12.94)		(72) Inventors; and (75) Inventors/Applicants (for US only): EATON, Dan, L. [US/US]; 75 Knight Drive, San Rafael, CA 94901 (US). DE SAUVAGE, Frederic, J. [BE/US]; 166 Beach Park Boulevard, Foster City, CA 94404 (US).																																																									
(30) Priority Data:		(74) Agents: WINTER, Daryl, B. et al; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).																																																									
<table border="0"> <tr> <td>08/176,553</td> <td>3 January 1994 (03.01.94)</td> <td>US</td> <td>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).</td> </tr> <tr> <td>08/185,607</td> <td>21 January 1994 (21.01.94)</td> <td>US</td> <td></td> </tr> <tr> <td>08/196,689</td> <td>15 February 1994 (15.02.94)</td> <td>US</td> <td></td> </tr> <tr> <td>08/223,263</td> <td>4 April 1994 (04.04.94)</td> <td>US</td> <td></td> </tr> <tr> <td>08/249,376</td> <td>25 May 1994 (25.05.94)</td> <td>US</td> <td></td> </tr> <tr> <td>08/348,657</td> <td>2 December 1994 (02.12.94)</td> <td>US</td> <td></td> </tr> <tr> <td>08/348,658</td> <td>2 December 1994 (02.12.94)</td> <td>US</td> <td></td> </tr> </table>		08/176,553	3 January 1994 (03.01.94)	US	(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).	08/185,607	21 January 1994 (21.01.94)	US		08/196,689	15 February 1994 (15.02.94)	US		08/223,263	4 April 1994 (04.04.94)	US		08/249,376	25 May 1994 (25.05.94)	US		08/348,657	2 December 1994 (02.12.94)	US		08/348,658	2 December 1994 (02.12.94)	US		<p>(60) Parent Applications or Grants (63) Related by Continuation</p> <table border="0"> <tr> <td>US</td> <td>08/249,376 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>25 May 1994 (25.05.94)</td> </tr> <tr> <td>US</td> <td>08/223,263 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>4 April 1994 (04.04.94)</td> </tr> <tr> <td>US</td> <td>08/196,689 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>15 February 1994 (15.02.94)</td> </tr> <tr> <td>US</td> <td>08/348,658 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>2 December 1994 (02.12.94)</td> </tr> <tr> <td>US</td> <td>08/185,607 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>21 January 1994 (21.01.94)</td> </tr> <tr> <td>US</td> <td>08/348,657 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>2 December 1994 (02.12.94)</td> </tr> <tr> <td>US</td> <td>08/176,553 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>3 January 1994 (03.01.94)</td> </tr> </table>		US	08/249,376 (CIP)	Filed on	25 May 1994 (25.05.94)	US	08/223,263 (CIP)	Filed on	4 April 1994 (04.04.94)	US	08/196,689 (CIP)	Filed on	15 February 1994 (15.02.94)	US	08/348,658 (CIP)	Filed on	2 December 1994 (02.12.94)	US	08/185,607 (CIP)	Filed on	21 January 1994 (21.01.94)	US	08/348,657 (CIP)	Filed on	2 December 1994 (02.12.94)	US	08/176,553 (CIP)	Filed on	3 January 1994 (03.01.94)
08/176,553	3 January 1994 (03.01.94)	US	(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).																																																								
08/185,607	21 January 1994 (21.01.94)	US																																																									
08/196,689	15 February 1994 (15.02.94)	US																																																									
08/223,263	4 April 1994 (04.04.94)	US																																																									
08/249,376	25 May 1994 (25.05.94)	US																																																									
08/348,657	2 December 1994 (02.12.94)	US																																																									
08/348,658	2 December 1994 (02.12.94)	US																																																									
US	08/249,376 (CIP)																																																										
Filed on	25 May 1994 (25.05.94)																																																										
US	08/223,263 (CIP)																																																										
Filed on	4 April 1994 (04.04.94)																																																										
US	08/196,689 (CIP)																																																										
Filed on	15 February 1994 (15.02.94)																																																										
US	08/348,658 (CIP)																																																										
Filed on	2 December 1994 (02.12.94)																																																										
US	08/185,607 (CIP)																																																										
Filed on	21 January 1994 (21.01.94)																																																										
US	08/348,657 (CIP)																																																										
Filed on	2 December 1994 (02.12.94)																																																										
US	08/176,553 (CIP)																																																										
Filed on	3 January 1994 (03.01.94)																																																										
<p>(54) Title: THROMBOPOIETIN (57) Abstract</p> <p>Isolated thrombopoietin (TPO), isolated DNA encoding TPO, and recombinant or synthetic methods of preparing and purifying TPO are disclosed. Various forms of TPO are shown to influence the replication, differentiation or maturation of blood cells, especially megakaryocytes and megakaryocyte progenitor cells. Accordingly, these compounds may be used for treatment of thrombocytopenia.</p>																																																											
<p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>																																																											

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NR	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## THROMBOPOIETIN

### FIELD OF THE INVENTION

This invention relates to the isolation, purification and recombinant or chemical synthesis of proteins that influence survival, proliferation, differentiation or maturation of hematopoietic cells, especially platelet progenitor cells. This invention specifically relates to the cloning and expression of nucleic acids encoding a protein ligand capable of binding to and activating *mpl*, a member of the cytokine receptor superfamily. This invention further relates to the use of these proteins alone or in combination with other cytokines to treat immune or hematopoietic disorders including thrombocytopenia.

### BACKGROUND OF THE INVENTION

#### I. The Hematopoietic System

The hematopoietic system produces the mature highly specialized blood cells known to be necessary for survival of all mammals. These mature cells include; erythrocytes, specialized to transport oxygen and carbon dioxide, T- and B-lymphocytes, responsible for cell- and antibody-mediated immune responses, platelets or thrombocytes, specialized to form blood clots, and granulocytes and macrophages, specialized as scavengers and as accessory cells to combat infection. Granulocytes are further subdivided into; neutrophils, eosinophils, basophils and mast cells, specialized cell types having discrete functions. Remarkably, all of these specialized mature blood cells are derived from a single common primitive cell type, referred to as the pluripotent (or totipotent) stem cell, found primarily in bone marrow (Dexter *et al.*, *Ann. Rev. Cell Biol.*, 3:423-441 [1987]).

The mature highly specialized blood cells must be produced in large numbers continuously throughout the life of a mammal. The vast majority of these specialized blood cells are destined to remain functionally active for only a few hours to weeks (Cronkite *et al.*, *Blood Cells*, 2:263-284 [1976]). Thus, continuous renewal of the mature blood cells, the primitive stem cells themselves, as well as any intermediate or lineage-committed progenitor cell lines lying between the primitive and mature cells, is necessary in order to maintain the normal steady state blood cell needs of the mammal.

At the heart of the hematopoietic system lies the pluripotent stem cell(s). These cells are relatively few in number and undergo self-renewal by proliferation to produce daughter stem cells or are transformed, in a series of differentiation steps,

into increasingly mature lineage-restricted progenitor cells, ultimately forming the highly specialized mature blood cell(s).

For example, certain multipotent progenitor cells, referred to as CFC-Mix, derived from stem cells undergo proliferation (self-renewal) and development to produce colonies containing all the different myeloid cells; erythrocytes, neutrophils, megakaryocytes (predecessors of platelets), macrophages, basophils, eosinophils, and mast cells. Other progenitor cells of the lymphoid lineage undergo proliferation and development into T-cells and B-cells.

Additionally, between the CFC-Mix progenitor cells and myeloid cells lie another rank of progenitor cells of intermediate commitment to their progeny. These lineage-restricted progenitor cells are classified on the basis of the progeny they produce. Thus, the known immediate predecessors of the myeloid cells are: erythroid colony-forming units (CFU-E) for erythrocytes, granulocyte/macrophage colony-forming cells (GM-CFC) for neutrophils and macrophages, megakaryocyte colony-forming cells (Meg-CFC) for megakaryocytes, eosinophil colony-forming cells (Eos-CFC) for eosinophils, and basophil colony-forming cells (Bas-CFC) for mast cells. Other intermediate predecessor cells between the pluripotent stem cells and mature blood cells are known (see below) or will likely be discovered having varying degrees of lineage-restriction and self-renewal capacity.

The underlying principal of the normal hematopoietic cell system appears to be decreased capacity for self-renewal as multipotency is lost and lineage-restriction and maturity is acquired. Thus, at one end of the hematopoietic cell spectrum lies the pluripotent stem cell possessing the capacity for self-renewal and differentiation into all the various lineage-specific committed progenitor cells. This capacity is the basis of bone marrow transplant therapy where primitive stem cells repopulate the entire hematopoietic cell system. At the other end of the spectrum lie the highly lineage-restricted progenitors and their progeny which have lost the ability of self-renewal but have acquired mature functional activity.

The proliferation and development of stem cells and lineage-restricted progenitor cells is carefully controlled by a variety of hematopoietic growth factors or cytokines. The role of these growth factors *in vivo* is complex and incompletely understood. Some growth factors, such as interleukin-3 (IL-3), are capable of stimulating both multipotent stem cells as well as committed progenitor cells of several lineages, including for example, megakaryocytes. Other factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) was initially thought to be restricted in its action to GM-CFC's. Later, however, it was discovered GM-CSF also influenced the proliferation and development of *interalia* megakaryocytes. Thus, IL-3 and GM-CSF were found to have overlapping biological activities, although with

different p tency. More recently, both interleukin-6 (IL-6) and interleukin-11 (IL-11), while having no apparent influence on meg-colony formation alone, act synergistically with IL-3 to stimulate maturation of megakaryocytes (Yonemura *et al.*, *Exp. Hematol.*, 20:1011-1016 [1992]).

5 Thus, hematopoietic growth factors may influence growth and differentiation of one or more lineages, may overlap with other growth factors in affecting a single progenitor cell line, or may act synergistically with other factors.

10 It also appears that hematopoietic growth factors can exhibit their effect at different stages of cell development from the totipotent stem cell through various committed lineage-restricted progenitors to the mature blood cell. For example, erythropoietin (epo) appears to promote proliferation only of mature erythroid progenitor cells. IL-3 appears to exert its effect earlier influencing primitive stem cells and intermediate lineage-restricted progenitor cells. Other growth factors such as stem cell factor (SCF) may influence even more primitive cell development.

15 It will be appreciated from the foregoing that novel hematopoietic growth factors that affect survival, proliferation, differentiation or maturation of any of the blood cells or predecessors thereof would be useful, especially to assist in the re-establishment of a diminished hematopoietic system caused by disease or after radiation- or chemo-therapy.

20

### II. Megakaryocytopoiesis - Platelet Production

Regulation of megakaryocytopoiesis and platelet production has been reviewed by: Mazur, *Exp. Hematol.*, 15:248 [1987] and Hoffman, *Blood*, 74:1196-1212 [1989]. Briefly, bone marrow pluripotent stem cells differentiate into megakaryocytic, erythrocytic, and myelocytic cell lines. It is believed there is a hierarchy of committed megakaryocytic progenitor cells between stem cells and megakaryocytes. At least three classes of megakaryocytic progenitor cells have been identified, namely: burst forming unit megakaryocytes (BFU-MK), colony-forming unit megakaryocytes (CFU-MK), and light density megakaryocyte progenitor cells (LD-CFU-MK). Megakaryocytic maturation itself is a continuum of development that has been separated into stages based on standard morphologic criteria. The earliest recognizable member of the megakaryocyte (MK or meg) family are the megakaryoblasts. These cells are initially 20 to 30  $\mu$ m in diameter having basophilic cytoplasm and a slightly irregular nucleus with loose, somewhat reticular chromatin and several nucleoli. Later, megakaryoblasts may contain up to 32 nuclei (polyploid), but the cytoplasm remains sparse and immature. As maturation proceeds, the nucleus becomes more lobulate and pyknotic, the cytoplasm increases in quantity and becomes more acidophilic and granular. The most mature cells of this family may give the

appearance of releasing platelets at their periphery. Normally, less than 10% of megakaryocytes are in the blast stage and more than 50% are mature. Arbitrary morphologic classifications commonly applied to the megakaryocyte series are megakaryoblast for the earliest form; promegakaryocyte or basophilic megakaryocyte for the intermediate form; and mature (acidophilic, granular, or platelet-producing) megakaryocyte for the late forms. The mature megakaryocyte extends filaments of cytoplasm into sinusoidal spaces where they detach and fragment into individual platelets (Williams *et al.*, *Hematology*, 1972).

Megakaryocytopoiesis is believed to involve several regulatory factors (Williams *et al.*, *Br. J. Haematol.*, 52:173 [1982] and Williams *et al.*, *J. Cell Physiol.*, 110:101 [1982]). The early level of megakaryocytopoiesis is postulated as being mitotic, concerned with cell proliferation and colony initiation from CFU-MK but is not affected by platelet count (Burstein *et al.*, *J. Cell Physiol.*, 109:333 [1981] and Kimura *et al.*, *Exp. Hematol.*, 13:1048 [1985]). The later stage of 15 maturation is non-mitotic, involved with nuclear polyploidization and cytoplasmic maturation and is probably regulated in a feedback mechanism by peripheral platelet number (Odell *et al.*, *Blood*, 48:765 [1976] and Ebbe *et al.*, *Blood*, 32:787 [1968]).

The existence of a distinct and specific megakaryocyte colony-stimulating factor (MK-CSF) has been disputed (Mazur, *Exp. Hematol.*, 15:340-350 [1987]). However most authors believe that a process so vital to survival as platelet production would be regulated by cytokine(s) exclusively responsible for this process. The hypothesis that megakaryocyte/platelet specific cytokine(s) exist has provided the basis for more than 30 years of search - but to date no such cytokine has been 25 purified, sequenced and established by assay as a unique MK-CSF (TPO).

Although it has been reported that MK-CSF's have been partly purified from experimentally produced thrombocytopenia (Hill *et al.*, *Exp. Hematol.*, 14:752 [1986]) and human embryonic kidney conditioned medium [CM] (McDonald *et al.*, *J. Lab. Clin. Med.*, 85:59 [1975]) and in man from a plastic anemia and idiopathic 30 thrombocytopenic purpura urinary extracts (Kawakita *et al.*, *Blood*, 6:556 [1983]) and plasma (Hoffman *et al.*, *J. Clin. Invest.*, 75:1174 [1985]), their physiological function is as yet unknown in most cases.

The conditioned medium of pokeweed mitogen-activated spleen cells (PWM-SpCM) and the murine myelomonocyte cell line WEHI-3 (WEHI-3CM) have been used 35 as megakaryocyte potentiators. PWM-SpCM contains factors enhancing CFU-MK growth (Metcalf *et al.*, *Proc. Natl. Acad. Sci., USA*, 72:1744-1748 [1975]; Quesenberry *et al.*, *Blood*, 65:214 [1985]; and Iscove, N.N., in *Hematopoietic Cell Differentiation, ICN-UCLA Symposia on Molecular and Cellular Biology*, Vol. 10, Golde

et al., eds. [New York, Academy Press] pp 37-52 [1978]), on of which is Interleukin-3 (IL-3), a multilineage colony stimulating factor (multi-CSF [Burstein, *Blood Cells*, 11:469 [1986]]). The other factors in this medium have not yet been identified and isolated. WEHI-3 is a murine myelomonocytic cell line secreting relatively large amounts of IL-3 and smaller amounts of GM-CSF. IL-3 has been found to potentiate the growth of a wide range of hematopoietic cells (Ihle et al., *J. Immunol.*, 13:282 [1983]). IL-3 has also been found to synergize with many of the known hematopoietic hormones or growth factors (Bartelmez et al., *J. Cell Physiol.*, 122:362-369 [1985] and Warren et al., *Cell*, 46:667-674 [1988]). Including both erythropoletin (EPO) and interleukin-1 (IL-1), in the induction of very early multipotential precursors and the formation of very large mixed hematopoietic colonies.

Other sources of megakaryocyte potentiators have been found in the conditioned media of murine lung, bone, macrophage cell lines, peritoneal exudate cells and human embryonic kidney cells. Despite certain conflicting data (Mazur, *Exp. Hematol.*, 15:340- 350 [1987]), there is some evidence (Geissler et al., *Br. J. Haematol.*, 60:233-238 [1985]) that activated T lymphocytes rather than monocytes play an enhancing role in megakaryocytopoiesis. These findings suggest that activated T-lymphocyte secretions such as interleukins may be regulatory factors in MK development (Geissler et al., *Exp. Hematol.*, 15:845-853 [1987]). A number of studies on megakaryocytopoiesis with purified erythropoletin EPO (Vainchenker et al., *Blood*, 54:940 [1979]; McLeod et al., *Nature*, 261:492-4 [1976]; and Williams et al., *Exp. Hematol.*, 12:734 [1984]) indicate that this hormone has an enhancing effect on MK colony formation. This has also been demonstrated in both serum-free and serum-containing cultures and in the absence of accessory cells (Williams et al., *Exp. Hematol.*, 12:734 [1984]). EPO was postulated to be involved more in the single and two-cell stage aspects of megakaryocytopoiesis as opposed to the effect of PWM-SpCM which was involved in the four-cell stage of megakaryocyte development. The interaction of all these factors on both early and late phases of megakaryocyte development remains to be elucidated.

Data produced from several laboratories suggests that the only multi-lineage factors that individually have MK-colony stimulating activity are GM-CSF and IL-3 and, to a lesser extent, the B-cell stimulating factor IL-6 (Ikebuchi et al., *Proc. Natl. Acad. Sci. USA*, 84:9035 [1987]). More recently, several authors have reported that IL-11 and leukemia inhibitory factor (LIF) act synergistically with IL-3 to increase megakaryocyte size and ploidy (Yonemura et al., *British Journal of Hematology*, 84:16-23 [1993]; Burstein et al., *J. Cell. Physiol.*, 153:305-312 [1992]; Metcalf et al., *Blood*, 76:50-56 [1990]; Metcalf et al., *Blood*, 77:2150-2153 [1991];

Bruno *et al.*, *Exp. Hematol.*, 19:378-381 [1991]; and Yonemura *et al.*, *Exp. Hematol.*, 20:1011-1016 [1992]).

Other documents of interest include: Eppstein *et al.*, U.S. Patent No. 4,962,091; Chong, U.S. Patent No. 4,879,111; Fernandes *et al.*, U.S. Patent No. 4,604,377; Wissler *et al.*, U.S. Patent No. 4,512,971; Gottlieb, U.S. Patent No. 4,468,379; Bennett *et al.*, U.S. Patent No. 5,215,895; Kogan *et al.*, U.S. Patent No. 5,250,732; Kimura *et al.*, *Eur. J. Immunol.*, 20(9):1927-1931 [1990]; Secor *et al.*, *J. of Immunol.*, 144(4):1484-1489 [1990]; Warren *et al.*, *J. of Immunol.*, 140(1):94-99 [1988]; Warren *et al.*, *Exp. Hematol.*, 17(11):1095-1089 [1989]; Bruno *et al.*, *Exp. Hematol.*, 17(10):1038-1043 [1989]; Tanikawa *et al.*, *Exp. Hematol.*, 17(8):883-888 [1989]; Koike *et al.*, *Blood*, 75(12):2286-2291 [1990]; Lotem, *Blood*, 75(5):1545-1551 [1989]; Rennick *et al.*, *Blood*, 73(7):1828-1835 [1989]; and Clutterbuck *et al.*, *Blood*, 73(6):1504-1512 [1989].

15

### III. Thrombocytopenia

Platelets are critical elements of the blood clotting mechanism. Depletion of the circulating level of platelets, called thrombocytopenia, occurs in various clinical conditions and disorders. Thrombocytopenia is commonly defined as a platelet count below 150 X 10<sup>9</sup> per liter. The major causes of thrombocytopenia can be broadly divided into three categories on the basis of platelet life span, namely; (1) impaired production of platelets by the bone marrow, (2) platelet sequestration in the spleen (splenomegaly), or (3) increased destruction of platelets in the peripheral circulation (e.g., autoimmune thrombocytopenia or chemo- and radiation-therapy). Additionally, in patients receiving large volumes of rapidly administered platelet-poor blood products, thrombocytopenia may develop due to dilution.

The clinical bleeding manifestations of thrombocytopenia depend on the severity of thrombocytopenia, its cause, and possible associated coagulation defects. In general, patients with platelet counts between 20 and 100 X 10<sup>9</sup> per liter are at risk of excessive post traumatic bleeding, while those with platelet counts below 20 X 10<sup>9</sup> per liter may bleed spontaneously. These latter patients are candidates for platelet transfusion with attendant immune and viral risk. For any given degree of thrombocytopenia, bleeding tends to be more severe when the cause is decreased production rather than increased destruction of platelets. In the latter situation, accelerated platelet turnover results in the circulation of younger, larger and hemostatically more effective platelets. Thrombocytopenia may result from a variety of disorders briefly described below. A more detailed description may be found in Schafner, A. I., "Thrombocytopenia and Disorders of Platelet Function," *Internal*

*Medicine*, 3rd Ed., John J. Hutton *et al.*, Eds., Little Brown and Co., Boston/Toronto/London [1990].

(a) Thrombocytopenia due to impaired platelet production

Causes of congenital thrombocytopenia include constitutional aplastic anemia

5 (Fanconi syndrome) and congenital amegakaryocytic thrombocytopenia, which may be associated with skeletal malformations. Acquired disorders of platelet production are caused by either hypoplasia of megakaryocytes or ineffective thrombopoiesis. Megakaryocytic hypoplasia can result from a variety of conditions, including marrow aplasia (including idiopathic forms or myelosuppression by chemotherapeutic agents 10 or radiation therapy), myelofibrosis, leukemia, and invasion of the bone marrow by metastatic tumor or granulomas. In some situations, toxins, infectious agents, or drugs may interfere with thrombopoiesis relatively selectively; examples include transient thrombocytopenias caused by alcohol and certain viral infections and mild thrombocytopenia associated with the administration of thiazide diuretics. Finally, 15 ineffective thrombopoiesis secondary to megaloblastic processes (folate or B<sub>12</sub> deficiency) can also cause thrombocytopenia, usually with coexisting anemia and leukopenia.

Current treatment of thrombocytopenias due to decreased platelet production depends on identification and reversal of the underlying cause of the bone marrow 20 failure. Platelet transfusions are usually reserved for patients with serious bleeding complications, or for coverage during surgical procedures, since isoimmunization may lead to refractoriness to further platelet transfusions. Mucosal bleeding resulting from severe thrombocytopenia may be ameliorated by the oral or intravenous administration of the antifibrinolytic agents. Thrombotic complications may develop, 25 however, if antifibrinolytic agents are used in patients with disseminated intravascular coagulation (DIC).

(b) Thrombocytopenia due to splenic sequestration

Splenomegaly due to any cause may be associated with mild to moderate thrombocytopenia. This is a largely passive process (hypersplenism) of splenic 30 platelet sequestration, in contrast to the active destruction of platelets by the spleen in cases of immunomediated thrombocytopenia discussed below. Although the most common cause of hypersplenism is congestive splenomegaly from portal hypertension due to alcoholic cirrhosis, other forms of congestive, infiltrative, or lymphoproliferative splenomegaly are also associated with thrombocytopenia. Platelet 35 counts generally do not fall below  $50 \times 10^9$  per liter as a result of hypersplenism alone.

## ( ) Thrombocytopenia due to nonimmune-mediated platelet destruction

Thrombocytopenia can result from the accelerated destruction of platelets by various nonimmunologic processes. Disorders of this type include disseminated intravascular coagulation, prosthetic intravascular devices, extra corporeal circulation of the blood, and thrombotic microangiopathies such as thrombotic thrombocytopenic purpura. In all of these situations, circulating platelets that are exposed to either artificial surfaces or abnormal vascular intima either are consumed at these sites or are damaged and then prematurely cleared by the reticuloendothelial system. Disease states or disorders in which disseminated intravascular coagulation (DIC) may arise are set forth in greater detail in Braunwald *et al.* (eds), *Harrison's Principles of Internal Medicine*, 11th Ed., p.1478, McGraw Hill [1987]. Intravascular prosthetic devices, including cardiac valves and intra-aortic balloons can cause a mild to moderate destructive thrombocytopenia and transient thrombocytopenia in patients undergoing cardiopulmonary bypass or hemodialysis may result from consumption or damage of platelets in the extra corporeal circuit.

## (d) Drug-induced immune thrombocytopenia

More than 100 drugs have been implicated in immunologically mediated thrombocytopenia. However, only quinidine, quinine, gold, sulfonamides, cephalothin, and heparin have been well characterized. Drug-induced thrombocytopenia is frequently very severe and typically occurs precipitously within days while patients are taking the sensitizing medication.

## (e) Immune (autoimmune) thrombocytopenic purpura (ITP)

ITP in adults is a chronic disease characterized by autoimmune platelet destruction. The autoantibody is usually IgG although other immunoglobulins have also been reported. Although the autoantibody of ITP has been found to be associated with platelet membrane GPIIb/IIIa, the platelet antigen specificity has not been identified in most cases. Extravascular destruction of sensitized platelets occurs in the reticuloendothelial system of the spleen and liver. Although over one-half of all cases of ITP are idiopathic, many patients have underlying rheumatic or autoimmune diseases (e.g., systemic lupus erythematosus) or lymphoproliferative disorders (e.g., chronic lymphocytic leukemia).

## (f) HIV-Induced ITP

ITP is an increasingly common complication of HIV infection (Morris *et al.*, *Ann. Intern. Med.*, 96:714-717 [1982]), and can occur at any stage of the disease progression, both in patients diagnosed with the Acquired Immune Deficiency Syndrome (AIDS), those with AIDS-related complex, and those with HIV Infection but without AIDS symptoms. HIV Infection is a transmissible disease ultimately characterized by a profound deficiency of cellular immune function as well as the

occurrence of opportunistic infection and malignancy. The primary immunologic abnormality resulting from infection by HIV is the progressive depletion and functional impairment of T lymphocytes expressing the CD4 cell surface glycoprotein (Lane *et al.*, *Ann. Rev. Immunol.*, 3:477 [1985]). The loss of CD4 helper/inducer T cell function probably underlies the profound defects in cellular and humoral immunity leading to the opportunistic infections and malignancies characteristic of AIDS (H. Lane *supra*).

Although the mechanism of HIV-associated ITP is unknown, it is believed to be different from the mechanism of ITP not associated with HIV infection. (Walsh *et al.*, 10 *N. Eng. J. Med.*, 311:635-639 [1984]; and Ratner, *Am. J. Med.*, 86:194-198 [1989]).

#### IV. Current Therapy for Thrombocytopenia

The therapeutic approach to the treatment of patients with thrombocytopenia is 15 dictated by the severity and urgency of the clinical situation. The treatment is similar for HIV-associated and non-HIV-related thrombocytopenia, and although a number of different therapeutic approaches have been used, the therapy remains controversial.

Platelet counts in patients diagnosed with thrombocytopenia have been successfully increased by glucocorticoid (e.g., prednisolone) therapy, however in most 20 patients, the response is incomplete, or relapse occurs when the glucocorticoid dose is reduced or its administration is discontinued. Based upon studies with patients having HIV-associated ITP, some investigators have suggested that glucocorticoid therapy may result in predisposition to AIDS. Glucocorticoids are usually administered if platelet count falls below  $20 \times 10^9/\text{liter}$  or when spontaneous bleeding occurs.

25 For patients refractory to glucocorticoids, the compound:

4-(2-chlorophenyl)-9-methyl-2-[3-(4-morpholinyl)-3-propanon-1-yl]6H-thieno[3,2,f][1,2,4]triazolo[4,3,a][1,4]diazepin (WEB 2086) has been successfully used to treat a severe case of non HIV-associated ITP. A patient having platelet counts of 37,000-58,000/ $\mu\text{l}$  was treated with WEB 2086 and after 30 1-2 weeks treatment platelet counts increased to 140,000-190,000/ $\mu\text{l}$ . (EP 361,077 and Lohman *et al.*, *Lancet*, 1147 [1988]).

Although the optimal treatment for acquired amegakaryocytic thrombocytopenia purpura (AATP) is uncertain, antithymocyte globulin (ATG), a horse antiserum to human thymus tissue, has been shown to produce prolonged complete remission 35 (Trimble *et al.*, *Am. J. Hematol.*, 37:126-127 [1991]). A recent report however, indicates that the hematopoietic effects of ATG are attributable to thimerosal, where presumably the protein acts as a mercury carrier (Panella *et al.*, *Cancer Research*, 50:4429-4435 [1990]).

Good results have been reported with splenectomy. Splenectomy removes the major site of platelet destruction and a major source of autoantibody production in many patients. This procedure results in prolonged treatment-free remissions in a large number of patients. However, since surgical procedures are generally to be avoided in immune compromised patients, splenectomy is recommended only in severe cases of thrombocytopenia (e.g. severe HIV-associated ITP), in patients who fail to respond to 2 to 3 weeks of glucocorticoid treatment, or do not achieve sustained response after discontinuation of glucocorticoid administration. Based upon current scientific knowledge, it is unclear whether splenectomy predisposes patients to AIDS.

10 In addition to prednisolone therapy and splenectomy, certain cytotoxic agents, e.g., vincristine, and azidothimidine (AZT, zidovudine) also show promise in treating HIV-induced ITP; however, the results are preliminary.

It will be appreciated from the foregoing that one way to treat thrombocytopenia would be to obtain an agent capable of accelerating the differentiation and maturation of megakaryocytes or precursors thereof into the platelet-producing form. Considerable efforts have been expended on identifying such an agent, commonly referred to as "thrombopoietin" (TPO). Other names for TPO commonly found in the literature include; thrombocytopoiesis stimulating factor (TSF), megakaryocyte colony-stimulating factor (MK-CSF), megakaryocyte-stimulating factor and megakaryocyte potentiator. TPO activity was observed as early as 1959 (Rak *et al.*, *Med. Exp.*, 1:125) and attempts to characterize and purify this agent have continued to the present day. While reports of partial purification of TPO-active polypeptides exist (see, for example, Tayrien *et al.*, *J. Biol. Chem.*, 262:3262 [1987] and Hoffman *et al.*, *J. Clin. Invest.* 75:1174 [1985]), others have postulated that TPO is not a discrete entity in its own right but rather is simply the polyfunctional manifestation of a known hormone (IL-3, Sparrow *et al.*, *Prog. Clin. Biol. Res.*, 215:123 [1986]). Regardless of its form or origin, a molecule possessing thrombopoietic activity would be of significant therapeutic value. Although no protein has been unambiguously identified as TPO, considerable interest surrounds the recent discovery that *mpl*, a putative cytokine receptor, may transduce a thrombopoietic signal.

#### V. *Mpl* is a Megakaryocytopoietic Cytokine Receptor

It is believed that the proliferation and maturation of hematopoietic cells is tightly regulated by factors that positively or negatively modulate pluripotential stem cell proliferation and multilineage differentiation. These effects are mediated through the high-affinity binding of extracellular protein factors to specific cell surface receptors. These cell surface receptors share considerable homology and are generally

classified as members of the cytokine receptor superfamily. Members of the superfamily include receptors for: IL-2 ( $\beta$  and  $\gamma$  chains) (Hatakeyama *et al.*, *Science*, 244:551-556 [1989]; Takeshita *et al.*, *Science*, 257:379-382 [1991]); IL-3 (Itoh *et al.*, *Science*, 247:324-328 [1990]; Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:5459-5463 [1990]; Kitamura *et al.*, *Cell*, 66:1165-1174 [1991a]; Kitamura *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:5082-5086 [1991b]), IL-4 (Mosley *et al.*, *Cell*, 59:335-348 [1989], IL-5 (Takaki *et al.*, *EMBO J.*, 9:4367-4374 [1990]; Tavernier *et al.*, *Cell*, 66:1175-1184 [1991]), IL-6 (Yamasaki *et al.*, *Science*, 241:825-828 [1988]; Hibi *et al.*, *Cell*, 63:1149-1157 [1990]), IL-7 (Goodwin *et al.*, *Cell*, 60:941-951 [1990]), IL-9 (Renault *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:5690-5694 [1992]), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gearing *et al.*, *EMBO J.*, 8:3667-3676 [1991]; Hayashida *et al.*, *Proc. Natl. Acad. Sci. USA*, 244:9655-9659 [1990]), granulocyte colony-stimulating factor (G-CSF) (Fukunaga *et al.*, *Cell*, 61:341-350 [1990a]; Fukunaga *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:8702-8706 [1990b]; Larsen *et al.*, *J. Exp. Med.*, 172:1559-1570 [1990]), EPO (D'Andrea *et al.*, *Cell*, 57:277-285 [1989]; Jones *et al.*, *Blood*, 76:31-35 [1990]), Leukemia inhibitory factor (LIF) (Gearing *et al.*, *EMBO J.*, 10:2839-2848 [1991]), oncostatin M (OSM) (Rose *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8641-8645 [1991]) and also receptors for prolactin (Boutin *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:7744-7748 [1988]; Edery *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:2112-2116 [1989]), growth hormone (GH) (Leung *et al.*, *Nature*, 330:537-543 [1987]) and ciliary neurotrophic factor (CNTF) (Davis *et al.*, *Science*, 253:59-63 [1991]).

Members of the cytokine receptor superfamily may be grouped into three functional categories (for review see Nicola *et al.*, *Cell*, 67:1-4 [1991]). The first class comprises single chain receptors, such as erythropoietin receptor (EPO-R) or granulocyte colony stimulating factor receptor (G-CSF-R), which bind ligand with high affinity via the extracellular domain and also generate an intracellular signal. A second class of receptors, so called  $\alpha$ -subunits, includes interleukin-6 receptor (IL6-R), granulocyte-macrophage colony stimulating factor receptor (GM-CSF-R), interleukin-3 receptor (IL3-R $\alpha$ ) and other members of the cytokine receptor superfamily. These  $\alpha$ -subunits bind ligand with low affinity but cannot transduce an intracellular signal. A high affinity receptor capable of signaling is generated by a heterodimer between an  $\alpha$ -subunit and a member of a third class of cytokine receptors, termed  $\beta$ -subunits, e.g.,  $\beta$ c, the common  $\beta$ -subunit for the three  $\alpha$ -subunits IL3-R $\alpha$  and GM-CSF-R.

Evidence that *mpl* is a member of the cytokine receptor superfamily comes from sequence homology (Gearing, *EMBO J.*, 8:3667-3676 [1988]; Bazan, *Proc.*

*Natl. Acad. Sci. USA*, 87:6834-6938 [1990]; Davis *et al.*, *Science*, 253:59-63 [1991] and Vigon *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:5640-5644 [1992]) and its ability to transduce proliferative signals.

Deduced protein sequence from molecular cloning of murine c-*mpl* reveals this 5 protein is homologous to other cytokine receptors. The extracellular domain contains 465 amino acid residues and is composed of two subdomains each with four highly conserved cysteines and a particular motif in the N-terminal subdomain and in the C-terminal subdomain. The ligand-binding extracellular domains are predicted to have similar double  $\beta$ -barrel fold structural geometries. This duplicated extracellular 10 domain is highly homologous to the signal transducing chain common to IL-3, IL-5 and GM-CSF receptors as well as the low-affinity binding domain of LIF (Vigon *et al.*, *Oncogene*, 8:2607-2615 [1993]). Thus *mpl* may belong to the low affinity ligand binding class of cytokine receptors.

A comparison of murine *mpl* and mature human *mpl* P, reveals these two 15 proteins show 81% sequence identity. More specifically, the N-terminus and C-terminus extracellular subdomains share 75% and 80% sequence identity respectively. The most conserved *mpl* region is the cytoplasmic domain showing 91% amino acid identity, with a sequence of 37 residues near the transmembrane domain being identical in both species. Accordingly, *mpl* is reported to be one of the most 20 conserved members of the cytokine receptor superfamily (Vigon *supra*).

Evidence that *mpl* is a functional receptor capable of transducing a proliferative signal comes from construction of chimeric receptors containing an extracellular domain from a cytokine receptor having high affinity for a known cytokine with the *mpl* cytoplasmic domain. Since no known ligand for *mpl* has been 25 reported, it was necessary to construct the chimeric high affinity ligand binding extracellular domain from a class one cytokine receptor such as IL-4R or G-CSFR. Vigon *et al.*, *supra* fused the extracellular domain of G-CSFR with both the transmembrane and cytoplasmic domain of c-*mpl*. An IL-3 dependent cell line, BAF/B03 (Ba/F3) was transfected with the G-CSFR/*mpl* chimera along with a full 30 length G-CSFR control. Cells transfected with the chimera grew equally well in the presence of cytokine IL-3 or G-CSF. Similarly, cells transfected with G-CSFR also grew well in either IL-3 or G-CSF. All cells died in the absence of growth factors. A similar experiment was conducted by Skoda *et al.*, *EMBO J.*, 12(7):2645-2653 35 [1993] in which both the extracellular and transmembrane domains of human IL-4 receptor (hIL-4-R) were fused to the murine *mpl* cytoplasmic domain, and transfected into a murine IL-3 dependent Ba/F3 cell line. Ba/F3 cells transfected with wild type hIL-4-R proliferated normally in the presence of either of the species specific IL-4 or IL-3. Ba/F3 cells transfected with hIL-4R/*mpl* proliferated

normally in the presence of hIL-4 (in the presence or absence of IL-3) demonstrating that in Ba/F3 cells the *mpl* cytoplasmic domain contains all the elements necessary to transduce a proliferative signal.

These chimeric experiments demonstrate the proliferation signaling capability 5 of the *mpl* cytoplasmic domain but are silent regarding whether the *mpl* extracellular domain can bind a ligand. These results are consistent with at least two possibilities, namely, *mpl* is a single chain (class one) receptor like EPO-R or G-CSFR or it is a signal transducing  $\beta$ -subunit (class three) requiring an  $\alpha$ -subunit like IL-3 (Skoda *et al. supra*).

10

#### VI. *Mpl* Ligand is a Thrombopoietin (TPO)

As described above, it has been suggested that serum contains a unique factor, sometimes referred to as thrombopoietin (TPO), that acts synergistically with various other cytokines to promote growth and maturation of megakaryocytes. No such natural 15 factor has ever been isolated from serum or any other source even though considerable effort has been expended by numerous groups. Even though it is not known whether *mpl* is capable of directly binding a megakaryocyte stimulating factor, recent experiments demonstrate that *mpl* is involved in proliferative signal transduction from a factor or factors found in the serum of patients with aplastic bone marrow 20 (Methia *et al.*, *Blood*, 82(5):1395-1401 [1993]).

20

Evidence that a unique serum colony-forming factor distinct from IL-1 $\alpha$ , IL-3, IL-4, IL-6, IL-11, SCF, EPO, G-CSF, and GM-CSF transduces a proliferative signal through *mpl* comes from examination of the distribution of c-*mpl* expression in primitive and committed hematopoietic cell lines and from *mpl* antisense studies in one 25 of these cell lines.

25

Using reverse transcriptase (RT)-PCR in immuno-purified human hematopoietic cells, Methia *et al.*, *supra* demonstrated that strong *mpl* mRNA messages were only found in CD34 $^{+}$  purified cells, megakaryocytes and platelets. CD34 $^{+}$  cells purified from bone marrow (BM) represents about 1% of all BM cells and are 30 enriched in primitive and committed progenitors of all lineages (e.g., erythroid, granulomacrophage, and megakaryocytic).

30

*Mpl* antisense oligodeoxynucleotides were shown to suppress megakaryocytic colony formation from the pluripotent CD34 $^{+}$  cells cultured in serum from patients with aplastic marrow (a rich source of megakaryocyte colony-stimulating activity [MK-CSA]). These same antisense oligodeoxynucleotides had no effect on erythroid or 35 granulomacrophage colony formation.

Whether *mpl* directly bound a ligand and whether the serum factor shown to cause megakaryocytopoiesis acted through *mpl* was still unknown. It had been

suggested, however, that if *mpl* did directly bind a ligand, its amino acid sequence was likely to be highly conserved and have species cross-reactivity owing to the considerable sequence identity between human and murine *mpl* extracellular domains (Vigon *et al.*, *supra* [1993]).

5

## VII. Objects

In view of the foregoing, it will be appreciated there is a current and continuing need in the art to isolate and identify molecules capable of stimulating proliferation, differentiation and maturation of hematopoietic cells, especially 10 megakaryocytes or their predecessors for therapeutic use in the treatment of thrombocytopenia. It is believed such a molecule is a *mpl* ligand and thus there exists a further need to isolate such ligand(s) to evaluate their role(s) in cell growth and differentiation.

Accordingly, it is an object of this invention to obtain a pharmaceutically pure 15 molecule capable of stimulating proliferation, differentiation and/or maturation of megakaryocytes into the mature platelet-producing form.

It is another object to provide the molecule in a form for therapeutic use in the treatment of a hematopoietic disorder, especially thrombocytopenia.

It is a further object of the present invention to isolate, purify and specifically 20 identify protein ligands capable of binding *in vivo* a cytokine superfamily receptor known as *mpl* and to transduce a proliferative signal.

It is still another object to provide nucleic acid molecules encoding such protein ligands and to use these nucleic acid molecules to produce *mpl* binding ligands in recombinant cell culture for diagnostic and therapeutic use.

25 It is yet another object to provide derivatives and modified forms of the protein ligands including amino acid sequence variants, variant glycoprotein forms and covalent derivatives thereof.

It is an additional object to provide fusion polypeptide forms combining a *mpl* ligand and a heterologous protein and covalent derivatives thereof.

30 It is still an additional object to provide variant polypeptide forms combining a *mpl* ligand with amino acid additions and substitutions from the EPO sequence to produce a protein capable of regulating proliferation and growth of both platelets and red blood cell progenitors.

It is yet an additional object to prepare immunogens for raising antibodies 35 against *mpl* ligands or fusion forms thereof, as well as to obtain antibodies capable of binding such ligands.

These and other objects of the invention will be apparent to the ordinary artisan upon consideration of the specification as a whole.

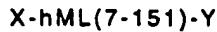
## SUMMARY OF THE INVENTION

The objects of the invention are achieved by providing an isolated mammalian megakaryocytopoietic proliferation and maturation promoting protein, denominated 5 the "mpl ligand" (ML) or "thrombopoietin" (TPO), capable of stimulating proliferation, maturation and/or differentiation of megakaryocytes into the mature platelet-producing form.

This substantially homogeneous protein may be purified from a natural source by a method comprising; (1) contacting a source plasma containing the *mpl* ligand 10 molecules to be purified with an immobilized receptor polypeptide, specifically *mpl* or a *mpl* fusion polypeptide immobilized on a support, under conditions whereby the *mpl* ligand molecules to be purified are selectively adsorbed onto the immobilized receptor polypeptide, (2) washing the immobilized receptor polypeptide and its support to remove non-adsorbed material, and (3) eluting the *mpl* ligand molecules from the 15 immobilized receptor polypeptide to which they are adsorbed with an elution buffer. Preferably the natural source is mammalian plasma or urine containing the *mpl* ligand. Optionally the mammal is aplastic and the immobilized receptor is a *mpl*-IgG fusion.

20 Optionally, the preferred megakaryocytopoietic proliferation and maturation promoting protein is an isolated substantially homogeneous *mpl* ligand polypeptide made by synthetic or recombinant means.

The "mpl ligand" polypeptide or "TPO" of this invention preferably has at least 25 70% overall sequence identity with the amino acid sequence of the highly purified substantially homogeneous porcine *mpl* ligand polypeptide and at least 80% sequence identity with the "EPO-domain" of the porcine *mpl* ligand polypeptide. Optionally, the *mpl* ligand of this invention is mature human *mpl* ligand (hML), having the mature amino acid sequence provided in Fig. 1 (SEQ ID NO: 1), or a variant or posttranscriptionally modified form thereof or a protein having about 80% sequence 30 identity with mature human *mpl* ligand. Optionally the *mpl* ligand variant is a fragment, especially an amino-terminus or "EPO-domain" fragment, of the mature human *mpl* ligand (hML). Preferably the amino terminus fragment retains substantially all of the human ML sequence between the first and forth cysteine residues but may contain substantial additions, deletions or substitutions outside that region. According to this embodiment, the fragment polypeptide may be represented by 35 the formula:



Where hML(7-151) represents the human TPO (hML) amino acid sequence from Cys<sup>7</sup> through Cys<sup>151</sup> inclusive; X represents the amino group of Cys<sup>7</sup> or one or more of the

amino-terminus amino acid residue(s) of the mature hML or amino acid residue extensions thereto such as Met, Tyr or leader sequences containing, for example, proteolytic cleavage sites (e.g. Factor Xa or thrombin); and Y represents the carboxy terminal group of Cys<sup>151</sup> or one or more carboxy-terminus amino acid residue(s) of the mature hML or extensions thereto.

5        Optionally the *mpl* ligand polypeptide or fragment thereof may be fused to a heterologous polypeptide (chimera). A preferred heterologous polypeptide is a cytokine, colony stimulating factor or interleukin or fragment thereof, especially kit-ligand (KL), IL-1, IL-3, IL-6, IL-11, EPO, GM-CSF or LIF. An optional preferred 10 heterologous polypeptide is an immunoglobulin chain, especially human IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD, IgM or fragment thereof, especially comprising the constant domain of an IgG heavy chain.

15        Another aspect of this invention provides a composition comprising an isolated *mpl* agonist that is biologically active and is preferably capable of stimulating the incorporation of labeled nucleotides (e.g., <sup>3</sup>H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl*. Optionally the *mpl* agonist is biologically active *mpl* ligand and is preferably capable of stimulating the incorporation of <sup>35</sup>S into circulating platelets in a mouse platelet rebound assay. Suitable *mpl* agonist include hML153, hML(R153A, R154A), hML2, hML3, hML4, 20 mML, mML2, mML3, pML, and pML2 or fragments thereof.

25        In another embodiment, this invention provides an isolated antibody capable of binding to the *mpl* ligand. The isolated antibody capable of binding to the *mpl* ligand may optionally be fused to a second polypeptide and the antibody or fusion thereof may be used to isolate and purify *mpl* ligand from a source as described above for immobilized *mpl*. In a further aspect of this embodiment, the invention provides a method for detecting the *mpl* ligand *in vitro* or *in vivo* comprising contacting the antibody with a sample, especially a serum sample, suspected of containing the ligand and detecting if binding has occurred.

30        In still further embodiments, the invention provides an isolated nucleic acid molecule, encoding the *mpl* ligand or fragments thereof, which nucleic acid molecule may optionally be labeled with a detectable moiety, and a nucleic acid molecule having a sequence that is complementary to, or hybridizes under moderate to highly stringent conditions with, a nucleic acid molecule having a sequence encoding a *mpl* ligand. Preferred nucleic acid molecules are those encoding human, porcine, and murine *mpl* 35 ligand, and include RNA and DNA, both genomic and cDNA. In a further aspect of this embodiment, the nucleic acid molecule is DNA encoding the *mpl* ligand and further comprises a replicable vector in which the DNA is operably linked to control sequences

recognized by a host transformed with the vector. Optionally the DNA is cDNA having the sequence provided in Fig. 1 5'-3' (SEQ ID NO: 2), 3'-5' or a fragment thereof. This aspect further includes host cells, preferably CHO cells, transformed with the vector and a method of using the DNA to effect production of *mpl* ligand, preferably 5 comprising expressing the cDNA encoding the *mpl* ligand in a culture of the transformed host cells and recovering the *mpl* ligand from the host cells or the host cell culture. The *mpl* ligand prepared in this manner is preferably human *mpl* ligand.

The invention further includes a method for treating a mammal having a hematopoietic disorder, especially thrombocytopenia, comprising administering a 10 therapeutically effective amount of a *mpl* ligand to the mammal. Optionally the *mpl* ligand is administered in combination with a cytokine, especially a colony stimulating factor or interleukin. Preferred colony stimulating factors or interleukins include; kit-ligand (KL), LIF, G-CSF, GM-CSF, M-CSF, EPO, IL-1, IL-3, IL-6, and IL-11.

The invention further includes a process for isolating and purifying TPO (ML) 15 from a TPO producing microorganism comprising:

- (1) disrupting or lysing cells containing TPO,
- (2) optionally separating soluble material from insoluble material containing TPO,
- (3) solubilizing TPO in the insoluble material with a solubilizing buffer,
- 20 (4) separating solubilized TPO from other soluble and insoluble material,
- (5) refolding TPO in a redox buffer, and
- (6) separating properly folded TPO from misfolded TPO.

The process provides for solubilizing the insoluble material containing TPO with a chaotropic agent where the chaotropic agent is selected from a salt of guanidine, 25 sodium thiocyanate, or urea. The process further provides that solubilized TPO is separated from other soluble and insoluble material by one or more steps selected from centrifugation, gel filtration and reverse phase chromatography. The refolding step of the process provides for a redox buffer containing both an oxidizing and reducing agent. Generally, the oxidizing agent is oxygen or a compound containing at 30 least one disulfide bond and the reducing agent is a compound containing at least one free sulphydryl. Preferably, the oxidizing agent is selected from oxidized glutathione(GSSG) and cystine and the reducing agent is selected from reduced glutathione(GSH) and cysteine. Most preferably the oxidizing agent is oxidized glutathione(GSSG) and the reducing agent is reduced glutathione(GSH). It is also 35 preferred that the molar ratio of the oxidizing agent is equal to or greater than that of the reducing agent. The redox buffer additionally contains a detergent, preferably selected from CHAPS and CHAPSO, present at a level of at least 1%. The redox buffer additionally contains NaCl preferably at a concentration range of about 0.1-0.5M, and

glycerol preferably at a concentration greater than 15%. The pH of the redox buffer preferably ranges from about pH 7.5-pH 9.0, and the refolding step is conducted at 4 degrees for 12-48hr. The refolding step produces biologically active TPO in which a disulfide bond is formed between the Cys nearest the amino-terminus with the Cys 5 nearest the carboxy-terminus of the EPO domain.

The invention further includes a process for purifying biologically active TPO from a microorganism comprising:

- (1) lysing at least the extracellular membrane of the microorganism,
- 10 (2) treating the lysate containing TPO with a chaotropic agent,
- (3) refolding the TPO, and
- (4) separating impurities and misfolded TPO from properly folded TPO.

#### 15 BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the deduced amino acid sequence (SEQ ID NO: 1) of human *mpl* ligand (hML) cDNA and the coding nucleotide sequence (SEQ ID NO: 2). Nucleotides are numbered at the beginning of each line. The 5' and 3' untranslated regions are indicated in lower case letters. Amino acid residues are numbered above the sequence 20 starting at Ser 1 of the mature *mpl* ligand (ML) protein sequence. The boundaries of presumed exon 3 are indicated by the arrows and the potential N-glycosylation sites are boxed. Cysteine residues are indicated by a dot above the sequence. The underlined sequence corresponds to the N-terminal sequence determined from *mpl* ligand purified from porcine plasma.

25 Fig. 2 shows the procedure used for the *mpl* ligand  $^3$ H-thymidine incorporation assay. To determine the presence of *mpl* ligand from various sources, the *mpl* P Ba/F3 cells were starved of IL-3 for 24 hours in a humidified incubator at 37°C in 5% CO<sub>2</sub> and air. Following IL-3 starvation the cells were plated out in 96 well culture dishes 30 with or without diluted samples and cultured for 24 hrs in a cell culture incubator. 20  $\mu$ l of serum free RPMI media containing 1  $\mu$ Ci of  $^3$ H-thymidine was added to each well for the last 6-8 hours. The cells were then harvested on 96 well filter plates and washed with water. The filters were then counted.

35 Fig. 3 shows the effect of pronase, DTT and heat on the ability of APP to stimulate Ba/F3-*mpl* cell proliferation. For pronase digestion of APP, pronase (Boehringer Mannheim) or bovine serum albumin was coupled to Affi-gel10 (Biorad) and incubated individually with APP for 18hrs. at 37°C. Subsequently, the resins were

removed by centrifugation and supernatants assayed. APP was also heated to 80°C for 4 min. or made 100 µM DTT followed by dialysis against PBS.

5 **Fig. 4** shows the elution of *mpl* ligand activity from Phenyl-Toyopearl, Blue-Sepharose and Ultralink-*mpl* columns. Fractions 4-8 from the *mpl* affinity column were the peak activity fractions eluted from the column.

10 **Fig. 5** shows the SDS-PAGE of eluted Ultralink-*mpl* fractions. To 200 µl of each fraction 2-8, 1 ml of acetone containing 1mM HCl at -20°C was added. After 3hrs. at -20°C samples were centrifuged and resultant pellets were washed 2x with acetone at -20°C. The acetone pellets were subsequently dissolved in 30 µl of SDS-solubilization buffer, made 100 µM DTT and heated at 90°C for 5 min. The samples were then resolved on a 4-20% SDS-polyacrylamide gel and proteins were visualized by silver staining.

15 **Fig. 6** shows elution of *mpl* ligand activity from SDS-PAGE. Fraction 6 from the *mpl*-affinity column was resolved on a 4-20% SDS-polyacrylamide gel under non-reducing conditions. Following electrophoresis the gel was sliced into 12 equal regions and electroeluted as described in the examples. The electroeluted samples were 20 dialyzed into PBS and assayed at a 1/20 dilution. The Mr standards used to calibrate the gel were Novex Mark 12 standards.

25 **Fig. 7** shows the effect of *mpl* ligand depleted APP on human megakaryocytopoiesis. *mpl* ligand depleted APP was made by passing 1 ml over a 1 ml *mpl*-affinity column (700 µg *mpl*-IgG/ml NHS-superose, Pharmacia). Human peripheral stem cell cultures were made 10% APP or 10% *mpl* ligand depleted APP and cultured for 12 days. Megakaryocytopoiesis was quantitated as described in the examples.

30 **Fig. 8** shows the effect of *mpl*-IgG on the stimulation of human megakaryocytopoiesis by APP. Human peripheral stem cell cultures were made 10% with APP and cultured for 12 days. At day 0, 2 and 4, *mpl*-IgG (0.5 µg) or ANP-R-IgG (0.5 µg) was added. After 12 days megakaryocytopoiesis was quantitated as described in the examples. The average of duplicate samples is graphed with the actual duplicate data in parenthesis.

35 **Fig. 9** shows both strands of a 390 bp fragment of human genomic DNA encoding the *mpl* ligand. The deduced amino acid sequence of "exon 3" (SEQ ID NO: 3), the coding sequence (SEQ ID NO: 4), and its compliment (SEQ ID NO: 5) are shown.

Fig. 10 shows deduced amino acid sequence of mature human *mpl* ligand (hML) (SEQ ID NO: 6) and mature human erythropoietin (hEPO) (SEQ ID NO: 7). The predicted amino acid sequence for the human *mpl* ligand is aligned with the human erythropoietin sequence. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Potential N-glycosylation sites are underlined with a plain line for the hML and with a broken line for hEPO. The two cysteines important for erythropoietin activity are indicated by a large dot.

Fig. 11 shows deduced amino acid sequence of mature human *mpl* ligand isoforms hML (SEQ ID NO: 6), hML2 (SEQ ID NO: 8), hML3 (SEQ ID NO: 9), and hML4 (SEQ ID NO: 10). Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes.

Figs. 12A, 12B and 12C show the effect of human *mpl* ligand on Ba/F3-*mpl* cell proliferation (A), *in vitro* human megakaryocytopoiesis quantitated using a radiolabeled murine IgG monoclonal antibody specific to the megakaryocyte glycoprotein GPIIbIIIa (B), and murine thrombopoiesis measured in a platelet rebound assay (C).

Two hundred ninety-three cells were transfected by the CaPO<sub>4</sub> method (Gorman, C in *DNA Cloning: A New Approach* 2:143-190 [1985]) with pRK5 vector alone, pRK5-hML or with pRK5-ML153 overnight (pRK5-ML153 was generated by introducing a stop codon after residue 153 of hML by PCR). Media was then conditioned for 36h and assayed for stimulation of cell proliferation of Ba/F3-*mpl* as described in Example 1 (A) or *in vitro* human megakaryocytopoiesis (B). Megakaryocytopoiesis was quantitated using a <sup>125</sup>I radiolabeled murine IgG monoclonal antibody (HP1-1D) to the megakaryocyte specific glycoprotein GPIIbIIIa as described (Grant et al., *Blood* 69:1334-1339 [1987]). The effect of partially purified recombinant ML (rML) on *in vivo* platelet production (C) was determined using the rebound thrombocytosis assay described by McDonald, T.P. *Proc. Soc. Exp. Biol. Med.* 144:1006-10012 (1973). Partially purified rML was prepared from 200ml of conditioned media containing the recombinant ML. The media was passed through a 2ml Blue-Separose column equilibrated in PBS and the column was washed with PBS and eluted with PBS containing 2M each of urea and NaCl. The active fraction was dialyzed into PBS and made 1mg/ml with endotoxin free BSA. The sample contained less than one unit of endotoxin /ml. Mice were injected with either 64,000, 32,000 or 16,000 units of rML or excipient alone. Each group consisted of six mice. The mean and standard deviation of each group is shown. p values were determined by a 2 tailed T-test comparing medians.

Fig. 13 compares the effect of human *mpl* ligand isoforms and variants in the Ba/F3-*mpl* cell proliferation assay. hML, mock, hML2, hML3, hML(R153A, R154A), and hML153 were assayed at various dilutions as described in Example 1.

5

Figs. 14A, 14B and 14C show the deduced amino acid sequence (SEQ ID NO: 1) of human *mpl* ligand (hML) or human TPO (hTPO) and the human genomic DNA coding sequence (SEQ ID NO: 11). Nucleotides and amino acid residues are numbered at the beginning of each line.

10

Fig. 15 shows a SDS-PAGE of purified 293-rhML332 and purified 293-rhML153.

15

Fig. 16 shows the nucleotide sequence: cDNA coding (SEQ ID NO: 12) and deduced amino acid sequence (SEQ ID NO: 13) of the open reading frame of a murine ML isoform. This mature murine *mpl* ligand isoform contains 331 amino acid residues, four fewer than the putative full length mML, and is therefore designated mML2. Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1. The potential N-glycosylation sites are underlined. Cysteine residues are indicated by a dot above the sequence.

20

Fig. 17 shows the cDNA sequence (SEQ ID NO: 14) and predicted protein sequence (SEQ ID NO: 15) of this murine ML isoform (mML). Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1. This mature murine *mpl* ligand isoform contains 335 amino acid residues and is believed to be the full length *mpl* ligand, designated mML. The signal sequence is indicated with a dashed underline and the likely cleavage point is denoted with an arrow. The 5' and 3' untranslated regions are indicated with lower case letters. The two deletions found as a result of alternative splicing (mML2 and mML3) are underlined. The four cysteine residues are indicated by a dot. The seven potential N-glycosylation sites are boxed.

30

Fig. 18 compares the deduced amino acid sequence of the human ML isoform hML3 (SEQ ID NO: 9) and a murine ML isoform designated mML3 (SEQ ID NO: 16). The predicted amino acid sequence for the human *mpl* ligand is aligned with the murine *mpl* ligand sequence. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Amino acids are numbered at the beginning of each line.

Fig. 19 compares the predicted amino acid sequences of mature ML isoforms from mouse-ML (SEQ ID NO: 17), porcine-ML (SEQ ID NO: 18) and human-ML (SEQ ID NO: 6). Amino acid sequences are aligned with gaps, indicated by dashes, introduced for optimal alignment. Amino acids are numbered at the beginning of each line with

5 Identical residues boxed. Potential N-glycosylation sites are indicated by a shaded box and cysteine residues are designated with a dot. The conserved di-basic amino acid motif that presents a potential protease cleavage site is underlined. The four amino acid deletion found to occur in all three species (ML2) is outlined with a bold box.

10 Fig. 20 shows the cDNA sequence (SEQ ID NO: 19) and predicted mature protein sequence (SEQ ID NO: 18) of a porcine ML isoform (pML). This porcine *mpl* ligand isoform contains 332 amino acid residues and is believed to be the full length porcine *mpl* ligand, designated pML. Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1.

15 Fig. 21 shows the cDNA sequence (SEQ ID NO: 20) and predicted mature protein sequence (SEQ ID NO: 21) of a porcine ML isoform (pML2). This porcine *mpl* ligand isoform contains 328 amino acid residues and is a four residues deletion form of the full length porcine *mpl* ligand, designated pML2. Nucleotides are numbered at the

20 beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1.

Fig. 22 compares the deduced amino acid sequence of the full length porcine ML isoform pML (SEQ ID NO: 18) and a porcine ML isoform designated pML2 (SEQ ID NO: 21). The predicted amino acid sequence for the pML is aligned with pML2 sequence. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Amino acids are numbered at the beginning of each line.

25 Fig. 23 shows the pertinent features of plasmid pSV15.ID.LL.MLORF ("full length" or TPO332) used to transfect host CHO-DP12 cells for production of CHO-rhTPO332.

30 Fig. 24 shows the pertinent features of plasmid pSV15.ID.LL.MLEPO-D ("truncated" or TPO153) used to transfect host CHO-DP12 cells for production of CHO-rhTPO153.

35 Figs. 25A, 25B, and 25C show the effect of *E. coli*-rhTPO(Met<sup>-1</sup>, 153) on platelets (A), red blood cells (B) and (C) white blood cells in normal mice. Two groups of 6 female C57 B6 mice were injected daily with either PBS buffer or 0.3 $\mu$ g *E. coli*-rhTPO(Met<sup>-1</sup>, 153) (100 $\mu$ l sc.). On day 0 and on days 3-7 40 $\mu$ l of blood was

tak n fr m th orbital sinus. This blood was immediately diluted in 10 ml of commercial diluant and complete blood counts were obtained on a Serrono Baker Hematology Analyzer 9018. The data are presented as means  $\pm$  Standard error of the mean.

5

**Figs. 26A, 26B and 26C** show the effect of *E. coli*-rhTPO(Met<sup>-1</sup>, 153) on platelets (A), red blood cells (B) and (C) white blood cells in sublethally irradiated mice. Two groups of 10 female C57 B6 mice were sublethally irradiated with 750 cGy of gamma radiation from a <sup>137</sup>Cs source and injected daily with either PBS buffer or 3.0 $\mu$ g *E. coli*-rhTPO(Met<sup>-1</sup>, 153) (100 $\mu$ l sc.). On day 0 and at subsequent intermediate time points 40 $\mu$ l of blood was taken from the orbital sinus. This blood was immediately diluted in 10 ml of commercial diluant and complete blood counts were obtained on a Serrono Baker Hematology Analyzer 9018. The data are presented as means  $\pm$  Standard error of the mean.

10

**Figs. 27A, 27B and 27C** show the effect of CHO-rhTPO332 on (A) platelets (thrombocytes), (B) red blood cells (erythrocytes) and (C) white blood cells (leukocytes) in normal mice. Two groups of 6 female C57 B6 mice were injected daily with either PBS buffer or 0.3 $\mu$ g CHO-rhTPO332 (100 $\mu$ l sc.). On day 0 and on 20 days 3-7 40 $\mu$ l of blood was taken from the orbital sinus. This blood was immediately diluted in 10 ml of commercial diluant and complete blood counts were obtained on a Serrono Baker Hematology Analyzer 9018. The data are presented as means  $\pm$  Standard error of the mean.

15

**Fig. 28** shows dose response curves for various forms of rhTPO obtained from various cell lines. Dose response curves were constructed to rhTPO from the following cell lines: hTPO332 from CHO (full length from Chinese hamster ovary cells); hTPOMet<sup>-1</sup> 153 (*E. coli*-derived truncated form with an N-terminal methionine); hTPO332 (full length TPO from human 293 cells); Met-less 155 *E-Coli* (the truncated form [rhTPO155] without the terminal methionine from *E. coli*). Groups of 6 female C57B6 mice were injected daily for 7 days with rhTPO depending upon group. Each day 40 $\mu$ l of blood was taken from the orbital sinus for complete blood counts. The data presented above are the maximal effects seen with the various treatments and with the exception of (met 153 *E-Coli*) this occurred on day 7 of treatment. In the aforementioned "met 153 *E-Coli*" group the maximal effect was seen on day 5. The data are presented as means  $\pm$  Standard error of the mean.

Fig. 29 shows dose response curves comparing the activity of full length and "clipped" forms of rhTPO produced in CHO cells with the truncated form from *E. coli*. Groups of 6 female C57B6 mice were injected daily with 0.3 $\mu$ g rhTPO of various types. On days 2-7 40 $\mu$ l of blood was taken from the orbital sinus for complete blood counts.

5 Treatment groups were TPO<sub>153</sub> the truncated form of TPO from *E. coli*; TPO332 (Mix fraction) Full length TPO containing approximately 80-90% full length and 10-20% clipped forms; TPO332(30K fraction) = purified clipped fraction from the original "mix" preparation; TPO332(70K fraction) = purified full length TPO fraction from the original "mix" preparation. The data are presented as means  $\pm$  Standard error of

10 the mean.

Fig. 30 is a cartoon showing the KIRA ELISA assay for measuring TPO. The figure shows the MPL/Rse.gD chimera and relevant parts of the parent receptors as well as the final construct (right portion of the figure) and a flow diagram (left portion of the

15 figure) showing relevant steps of the assay.

Fig. 31 is a flow chart for the KIRA ELISA assay showing each step in the procedure.

Figs. 32A-32L provide the nucleotide sequence (SEQ ID NO: 22) of the pSVI17.ID.LL expression vector used for expression of Rse.gD in Example 17.

Fig. 33 is a schematic representation of the preparation of plasmid pMP1.

Fig. 34 is a schematic representation of the preparation of plasmid pMP21.

25

Fig. 35 is a schematic representation of the preparation of plasmid pMP151.

Fig. 36 is a schematic representation of the preparation of plasmid pMP202.

30 Fig. 37 is a schematic representation of the preparation of plasmid pMP172.

Fig. 38 is a schematic representation of the preparation of plasmid pMP210.

35 Fig. 39 is a table of the five best expressing TPO clones from the pMP210 plasmid bank (SEQ ID NOS: 23, 24, 25, 26, 27 and 28).

Fig. 40 is a schematic representation of the preparation of plasmid pMP41.

Fig. 41 is a schematic representation of the preparation of plasmid pMP57.

Fig. 42 is a schematic representation of the preparation of plasmid pMP251.

## 5 DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims.

"Chaotropic agent" refers to a compound which, in aqueous solution and in 10 suitable concentrations, can cause a change in the spatial configuration or conformation of a protein by at least partially disrupting the forces responsible for maintaining the normal secondary and tertiary structure of the protein. Such compounds include, for example, urea, guanidine-HCl, and sodium thiocyanate. High concentrations, usually 4-9M, of these compounds are normally required to exert the 15 conformational effect on proteins.

"Cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone, insulin-like growth factors, human growth hormone, 20 N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and leutinizing hormone (LH), hematopoietic growth factor, hepatic growth factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor- $\alpha$  25 (TNF- $\alpha$  and TNF- $\beta$ ) mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, nerve growth factors such as NGF- $\beta$ , platelet-growth factor, transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ , insulin-like growth factor-I and -II, erythropoietin (EPO), osteoinductive factors, interferons such as interferon- $\alpha$ , - $\beta$ , and - $\gamma$ , colony 30 stimulating factors (CSFs) such as macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF), interleukins (IL's) such as IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12 and other polypeptide factors including LIF, SCF, and kit-ligand. As used herein the foregoing terms are meant to include proteins from natural sources or from 35 recombinant cell culture. Similarly, the terms are intended to include biologically active equivalents; e.g., differing in amino acid sequence by one or more amino acids or in type or extent of glycosylation.

"*mpl* ligand", "*mpl* ligand polypeptide", "ML", "thrombopoietin" or "TPO" are used interchangeably herein and comprise any polypeptide that possesses the property of binding to *mpl*, a member of the cytokine receptor superfamily, and having a biological property of the ML as defined below. An exemplary biological property is

5 the ability to stimulate the incorporation of labeled nucleotides (e.g.,  $^3$ H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl* P. Another exemplary biological property is the ability to stimulate the incorporation of  $^{35}$ S into circulating platelets in a mouse platelet rebound assay. This definition encompasses the polypeptide isolated from a *mpl* ligand source such as aplastic porcine plasma

10 described herein or from another source, such as another animal species, including humans or prepared by recombinant or synthetic methods and includes variant forms including functional derivatives, fragments, alleles, isoforms and analogues thereof.

A "*mpl* ligand fragment" or "TPO fragment" is a portion of a naturally occurring mature full length *mpl* ligand or TPO sequence having one or more amino acid residues or carbohydrate units deleted. The deleted amino acid residue(s) may occur anywhere in the peptide including at either the N-terminal or C-terminal end or internally. The fragment will share at least one biological property in common with *mpl* ligand. *Mpl* ligand fragments typically will have a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues that are identical to the sequences of

15 the *mpl* ligand isolated from a mammal including the ligand isolated from aplastic porcine plasma or the human or murine ligand, especially the EPO-domain thereof.

20 Representative examples of N-terminal fragments are hML153 or TPO(Met<sup>11</sup>-153).

"*Mpl* ligand variants" or "*mpl* ligand sequence variants" as defined herein means a biologically active *mpl* ligand as defined below having less than 100% sequence identity with the *mpl* ligand isolated from recombinant cell culture or aplastic porcine plasma or the human ligand having the deduced sequence described in Fig. 1 (SEQ ID NO: 1). Ordinarily, a biologically active *mpl* ligand variant will have an amino acid sequence having at least about 70% amino acid sequence identity with the *mpl* ligand isolated from aplastic porcine plasma or the mature murine or human

25 ligand or fragments thereof (see Fig. 1 [SEQ ID NO: 1]), preferably at least about 75%, more preferably at least about 80%, still more preferably at least about 85%, even more preferably at least about 90%, and most preferably at least about 95%.

A "chimeric *mpl* ligand" is a polypeptide comprising full length *mpl* ligand or one or more fragments thereof fused or bonded to a second heterologous polypeptide or

30 one or more fragments thereof. The chimera will share at least one biological property in common with *mpl* ligand. The second polypeptide will typically be a cytokine, immunoglobin or fragment thereof.

"Isolated *mpl* ligand", "highly purified *mpl* ligand" and "substantially homogeneous *mpl* ligand" are used interchangeably and mean a *mpl* ligand that has been purified from a *mpl* ligand source or has been prepared by recombinant or synthetic methods and is sufficiently free of other peptides or proteins (1) to obtain at least 15  
5 and preferably 20 amino acid residues of the N-terminal or of an internal amino acid sequence by using a spinning cup sequenator or the best commercially available amino acid sequenator marketed or as modified by published methods as of the filing date of this application, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Homogeneity here means  
10 less than about 5% contamination with other source proteins.

"Biological property" when used in conjunction with either the "*mpl* ligand" or "Isolated *mpl* ligand" means having thrombopoietic activity or having an *in vivo* effector or antigenic function or activity that is directly or indirectly caused or performed by a *mpl* ligand (whether in its native or denatured conformation) or a  
15 fragment thereof. Effector functions include *mpl* binding and any carrier binding activity, agonism or antagonism of *mpl*, especially transduction of a proliferative signal including replication, DNA regulatory function, modulation of the biological activity of other cytokines, receptor (especially cytokine) activation, deactivation, up- or down regulation, cell growth or differentiation and the like. An antigenic  
20 function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the native *mpl* ligand. The principal antigenic function of a *mpl* ligand polypeptide is that it binds with an affinity of at least about 10<sup>6</sup> l/mole to an antibody raised against the *mpl* ligand isolated from aplastic porcine plasma. Ordinarily, the polypeptide binds with an affinity of at least about 10<sup>7</sup>  
25 l/mole. Most preferably, the antigenically active *mpl* ligand polypeptide is a polypeptide that binds to an antibody raised against the *mpl* ligand having one of the above described effector functions. The antibodies used to define "biologically activity" are rabbit polyclonal antibodies raised by formulating the *mpl* ligand isolated from recombinant cell culture or aplastic porcine plasma in Freund's complete adjuvant,  
30 subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of *mpl* ligand antibody plateaus.

"Biologically active" when used in conjunction with either the "*mpl* ligand" or "Isolated *mpl* ligand" means a *mpl* ligand or polypeptide that exhibits thrombopoietic  
35 activity or shares an effector function of the *mpl* ligand isolated from aplastic porcine plasma or expressed in recombinant cell culture described herein. A principal known effector function of the *mpl* ligand or polypeptide herein is binding to *mpl* and stimulating the incorporation of labeled nucleotides (<sup>3</sup>H-thymidine) into the DNA of

IL-3 dependent Ba/F3 cells transfected with human *mpl* P. Another known effector function of the *mpl* ligand or polypeptide herein is the ability to stimulate the incorporation of  $^{35}\text{S}$  into circulating platelets in a mouse platelet rebound assay. Yet another known effector function of *mpl* ligand is the ability to stimulate *in vitro* 5 human megakaryocytopoiesis that may be quantitated by using a radio labeled monoclonal antibody specific to the megakaryocyte glycoprotein GPIIbIIIa.

"Percent amino acid sequence identity" with respect to the *mpl* ligand sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the *mpl* ligand sequence isolated from aplastic 10 porcine plasma or the murine or human ligand having the deduced amino acid sequence described in Fig. 1 (SEQ ID NO: 1), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the *mpl* ligand 15 sequence shall be construed as affecting sequence identity or homology. Thus exemplary biologically active *mpl* ligand polypeptides considered to have identical sequences include; prepro-*mpl* ligand, pro-*mpl* ligand, and mature *mpl* ligand.

"*Mpl* ligand microsequencing" may be accomplished by any appropriate standard procedure provided the procedure is sensitive enough. In one such method, 20 highly purified polypeptide obtained from SDS gels or from a final HPLC step are sequenced directly by automated Edman (phenyl isothiocyanate) degradation using a model 470A Applied Biosystems gas phase sequencer equipped with a 120A phenylthiohydantion (PTH) amino acid analyzer. Additionally, *mpl* ligand fragments prepared by chemical (e.g., CNBr, hydroxylamine, 2-nitro-5-thiocyanobenzoate) or 25 enzymatic (e.g., trypsin, clostrypain, staphylococcal protease) digestion followed by fragment purification (e.g., HPLC) may be similarly sequenced. PTH amino acids are analyzed using the ChromPerfect data system (Justice Innovations, Palo Alto, CA). Sequence interpretation is performed on a VAX 11/785 Digital Equipment Co. computer as described by Henzel *et al.*, *J. Chromatography*, 404:41-52 [1987]. 30 Optionally, aliquots of HPLC fractions may be electrophoresed on 5-20% SDS-PAGE, electrotransferred to a PVDF membrane (ProBlott, AIB, Foster City, CA) and stained with Coomassie Brilliant Blue (Matsurdiara, *J. Biol. Chem.*, 262:10035-10038 [1987]. A specific protein identified by the stain is excised from the blot and N-terminal sequencing is carried out with the gas phase sequenator described above. For 35 internal protein sequences, HPLC fractions are dried under vacuum (SpeedVac), resuspended in appropriate buffers, and digested with cyanogen bromide, the Lys-specific enzyme Lys-C (Wako Chemicals, Richmond, VA), or Asp-N (Boehringer Mannheim, Indianapolis, IN). After digestion, the resultant peptides are sequenced as a

mixture or after HPLC resolution on a C4 column developed with a propanol gradient in 0.1% TFA prior to gas phase sequencing.

"Thrombocytopenia" is defined as a platelet count below  $150 \times 10^9$  per liter of blood.

5 "Thrombopoietic activity" is defined as biological activity that consists of accelerating the proliferation, differentiation and/or maturation of megakaryocytes or megakaryocyte precursors into the platelet producing form of these cells. This activity may be measured in various assays including an *in vivo* mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured 10 by an anti-platelet immunoassay (anti-GPII<sub>b</sub>III<sub>a</sub>) for a human leukemia megakaryoblastic cell line (CMK), and induction of polyploidization in a megakaryoblastic cell line (DAMI).

15 "Thrombopoietin" (TPO) is defined as a compound having thrombopoietic activity or being capable of increasing serum platelet counts in a mammal. TPO is preferably capable of increasing endogenous platelet counts by at least 10%, more preferably by 50%, and most preferably capable of elevating platelet counts in a human to greater than  $150 \times 10^9$  per liter of blood.

20 "Isolated *mpl* ligand nucleic acid" is RNA or DNA containing greater than 16 and preferably 20 or more sequential nucleotide bases that encode biologically active *mpl* ligand or a fragment thereof, is complementary to the RNA or DNA, or hybridizes to the RNA or DNA and remains stably bound under moderate to stringent conditions. This RNA or DNA is free from at least one contaminating source nucleic acid with which it is normally associated in the natural source and preferably substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source 25 nucleic acid with which it is normally associated" includes the case where the nucleic acid is present in the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell. An example of isolated *mpl* ligand nucleic acid is RNA or DNA that encodes a biologically active *mpl* ligand sharing at least 75% sequence identity, more preferably at least 30 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95% sequence identity with the human, murine or porcine *mpl* ligand.

35 "Control sequences" when referring to expression means DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" when referring to nucleic acids means that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Exogenous" when referring to an element means a nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

"Cell," "cell line," and "cell culture" are used interchangeably herein and such designations include all progeny of a cell or cell line. Thus, for example, terms like "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are autonomously replicating circular DNA molecules possessing independent origins of replication and are designated herein by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from such available plasmids in accordance with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Restriction enzyme digestion" when referring to DNA means catalytic cleavage of internal phosphodiester bonds of DNA with an enzyme that acts only at certain locations or sites in the DNA sequence. Such enzymes are called "restriction endonucleases". Each restriction endonuclease recognizes a specific DNA sequence called a "restriction site" that exhibits two-fold symmetry. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital

letter foll w d by other letters representing the microorganism from which each restriction enzyme originally was obtained and th n a number designating the particular enzyme. In general, about 1  $\mu$ g of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20  $\mu$ l of buffer solution. Appropriate buffers and 5 substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a 10 restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction-cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents 15 for dephosphorylation are conventional as described in sections 1.56-1.61 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* [New York: Cold Spring Harbor Laboratory Press, 1989].

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, 20 identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, *Nucleic Acids Res.*, 9:6103-6114 [1981], and Goeddel *et al.*, *Nucleic Acids Res.*, 8:4057 [1980].

25 "Southern analysis" or "Southern blotting" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and 30 transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook *et al.*, *supra*.

35 "Northern analysis" or "Northern blotting" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as  $^{32}P$ , or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe,

using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, *supra*.

"Ligation" is the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol-chloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 µg of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large- and small-scale plasmid preparations described in sections 1.25-1.33 of Sambrook *et al.*, *supra*. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid-phase techniques such as described in EP 266,032 published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, *Nucl. Acids Res.*, 14:5399-5407 [1986]). Further methods include the polymerase chain reaction defined below and other autoprimer methods and oligonucleotide syntheses on solid supports. All of these methods are described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, 28:716-734 (1989). These methods are used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue. The oligonucleotides are then purified on polyacrylamide gels.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be 5 available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage 10 or plasmid sequences, etc. See generally Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 51:263 [1987]; Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase 15 to amplify or generate a specific piece of nucleic acid.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDODSO<sub>4</sub> (SDS) at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum 20 albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with 25 washes at 42°C in 0.2 x SSC and 0.1% SDS.

"Moderately stringent conditions" are described in Sambrook *et al.*, *supra*, and include the use of a washing solution and hybridization conditions (e.g., temperature, ionic strength, and %SDS) less stringent than described above. An example of moderately stringent conditions are conditions such as overnight incubation at 37°C in 30 a solution comprising: 20% formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 X Denhardt's solution, 10% dextran sulfate, and 20 µl/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 X SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength etc. as necessary to accommodate factors such as probe 35 length and the like.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like

molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies and immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ( $V_H$ ) followed by a number of constant domains. Each light chain has a variable domain at one end ( $V_L$ ) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, *J. Mol. Biol.*, **186**:651-663 [1985]; Novotny and Haber, *Proc. Natl. Acad. Sci. USA*, **82**:4592-4596 [1985]).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a  $\beta$ -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institute of Health, Bethesda, MD [1987]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen binding fragments, called "Fab" fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the  $V_H$ - $V_L$  dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other, chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda ( $\lambda$ ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called  $\alpha$ , delta, epsilon,  $\gamma$ , and  $\mu$ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (e.g., Fab,  $F(ab')_2$ , and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each

monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 10 4,816,567 [Cabilly *et al.*]).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of 15 the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567 (Cabilly *et al.*); and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 [1984]).

20 "Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a 25 complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found 30 neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the 35 FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones

*et al.*, *Nature*, 321:522-525 [1986]; Reichmann *et al.*, *Nature*, 332:323-329 [1988]; and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 [1992]).

"Non-immunogenic in a human" means that upon contacting the polypeptide in a pharmaceutically acceptable carrier and in a therapeutically effective amount with the 5 appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide is demonstratable upon the second administration of the polypeptide after an appropriate latent period (e.g., 8 to 14 days).

### II. Preferred Embodiments of the Invention

10 Preferred polypeptides of this invention are substantially homogeneous polypeptide(s), referred to as *mpl* ligand(s) or thrombopoletin (TPO), that possess the property of binding to *mpl*, a member of the receptor cytokine superfamily, and having the biological property of stimulating the incorporation of labeled nucleotides (<sup>3</sup>H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human 15 *mpl*. More preferred *mpl* ligand(s) are isolated mammalian protein(s) having hematopoietic, especially megakaryocytopoietic or thrombocytopoietic activity - namely, being capable of stimulating proliferation, maturation and/or differentiation of immature megakaryocytes or their predecessors into the mature platelet-producing form. Most preferred polypeptides of this invention are human *mpl* ligand(s) 20 including fragments thereof having hematopoietic, megakaryocytopoietic or thrombopoietic activity. Optionally these human *mpl* ligand(s) lack glycosylation. Other preferred human *mpl* ligands are the "EPO-domain" of hML referred to as hML153 or hTPO153, a truncated form of hML referred to as hML245 or hTPO245 and the 25 mature full length polypeptide having the amino acid sequence shown in Fig. 1 (SEQ ID NO: 1), referred to as hML, hML332 or hTPO332 and the biologically active substitutional variant hML(R153A, R154A).

Optional preferred polypeptides of this invention are biologically or immunologically active *mpl* ligand variants selected from hML2, hML3, hML4, mML, mML2, mML3, pML and pML2.

30 Optional preferred polypeptides of this invention are biologically active *mpl* ligand variant(s) that have an amino acid sequence having at least 70% amino acid sequence identity with the human *mpl* ligand (see Fig. 1 [SEQ ID NO: 1]), the murine *mpl* ligand (see Fig. 16 [SEQ ID NOS: 12 & 13]), the recombinant porcine *mpl* ligand (see Fig. 19 [SEQ ID NO: 18]) or the porcine *mpl* ligand isolated from aplastic 35 porcine plasma, preferably at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95%.

The *mpl* ligand isolated from aplastic porcine plasma has the following characteristics:

(1) The partially purified ligand elutes from a gel filtration column run in either PBS, PBS containing 0.1% SDS or PBS containing 4M MgCl<sub>2</sub> with Mr of 60,000-70,000;

(2) The ligand's activity is destroyed by pronase;

(3) The ligand is stable to low pH (2.5), SDS to 0.1%, and 2M urea;

(4) The ligand is a glycoprotein, based on its binding to a variety of lectin columns;

(5) The highly purified ligand elutes from non-reduced SDS-PAGE with a Mr of 25,000-35,000. Smaller amounts of activity also elute with Mr of ~18,000-22,000 and 60,000;

(6) The highly purified ligand resolves on reduced SDS-PAGE as a doublet with Mr of 28,000 and 31,000;

(7) The amino-terminal sequence of the 18,000-22,000, 28,000 and 31,000 bands is the same - SPAPPACDPRLLNKLLRDDHVHLGR (SEQ ID NO: 29); and

(8) The ligand binds and elutes from the following affinity columns

Blue-Sepharose,

CM Blue-Sepharose,

MONO-Q,

MONO-S,

Lentil lectin-Sepharose,

WGA-Sepharose,

Con A-Sepharose,

Ether 650m Toyopearl,

Butyl 650 m Toyopearl,

Phenyl 650m Toyopearl, and

Phenyl-Sepharose.

More preferred *mp1* ligand polypeptides are those encoded by human genomic or cDNA having an amino acid sequence described in Fig. 1 (SEQ ID NO: 1).

Other preferred naturally occurring biologically active *mpl* ligand polypeptides of this invention include prepro-*mpl* ligand, pro-*mpl* ligand, mature *mpl* ligand, *mpl* ligand fragments and glycosylation variants thereof.

Still other preferred polypeptides of this invention include *mpl* ligand sequence variants and chimeras. Ordinarily, preferred *mpl* ligand sequence variants and chimeras are biologically active *mpl* ligand variants that have an amino acid sequence having at least 70% amino acid sequence identity with the human *mpl* ligand or the *mpl* ligand isolated from aplastic porcine plasma, preferably at least 75%, more

preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95%. An exemplary preferred *mpl* ligand variant is a N-terminal domain hML variant (referred to as the "EPO-domain" because of its sequence homology to erythropoietin). The preferred hML EPO-domain 5 comprises about the first 153 amino acid residues of mature hML and is referred to as hML153. An optionally preferred hML sequence variant comprises one in which one or more of the basic or dibasic amino acid residue(s) in the C-terminal domain is substituted with a non-basic amino acid residue(s) (e.g., hydrophobic, neutral, acidic, aromatic, Gly, Pro and the like). A preferred hML C-terminal domain sequence variant 10 comprises one in which Arg residues 153 and 154 are replaced with Ala residues. This variant is referred to as hML332(R153A, R154A). An alternative preferred hML variant comprises either hML332 or hML153 in which amino residues 111-114 (QLPP or LPPQ) are deleted or replaced with a different tetrapeptide sequence (e.g. AGAG or the like). The foregoing deletion mutants are referred to as  $\Delta 4$ hML332 or 15  $\Delta 4$ hML153.

A preferred chimera is a fusion between *mpl* ligand or fragment (defined below) thereof with a heterologous polypeptide or fragment thereof. For example, hML153 may be fused to an IgG fragment to improve serum half-life or to IL-3, G-CSF or EPO to produce a molecule with enhanced thrombopoietic or chimeric 20 hematopoietic activity.

An alternative preferred human *mpl* ligand chimera is a "ML-EPO domain 25 chimera" that consists of the N-terminus 153 to 157 hML residues substituted with one or more, but not all, of the human EPO residues approximately aligned as shown in Fig. 10 (SEQ ID NO: 7). In this embodiment, the hML chimera would be about 153-166 residues in length in which individual or blocks of residues from the human EPO sequence are added or substituted into the hML sequence at positions corresponding to the alignment shown in Fig. 10 (SEQ ID NO: 6). Exemplary block sequence inserts into the N-terminus portion of hML would include one or more of the N-glycosylation sites at positions (EPO) 24-27, 38-40, and 83-85; one or more of the four 30 predicted amphipathic  $\alpha$ -helical bundles at positions (EPO) 9-22, 59-76, 90-107, and 132-152; and other highly conserved regions including the N-terminus and C-terminus regions and residue positions (EPO) 44-52 (see e.g., Wen et al., *Blood*, 82:1507-1516 [1993] and Boissel et al., *J. Biol. Chem.*, 268(21):15983-15993 [1993]). It is contemplated this "ML-EPO domain chimera" will have mixed 35 thrombopoietic-erythropoietic (TEPO) biological activity.

Other preferred polypeptides of this invention include *mpl* ligand fragments having a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues that are identical to the sequences of the *mpl* ligand isolated from aplastic

porcine plasma or the human *mpl* ligand described herein (see e.g. Table 14, Example 24). A preferred *mpl* ligand fragment is human ML[1-X] where X is 153, 164, 191, 205, 207, 217, 229, or 245 (see Fig. 1 [SEQ ID NO: 1] for the sequence of residues 1-X). Other preferred *mpl* ligand fragments include those produced as a result of chemical or enzymatic hydrolysis or digestion of the purified ligand.

Another preferred aspect of the invention is a method for purifying *mpl* ligand molecules comprises contacting a *mpl* ligand source containing the *mpl* ligand molecules with an immobilized receptor polypeptide, specifically *mpl* or a *mpl* fusion polypeptide, under conditions whereby the *mpl* ligand molecules to be purified are selectively adsorbed onto the immobilized receptor polypeptide, washing the immobilized support to remove non-adsorbed material, and eluting the molecules to be purified from the immobilized receptor polypeptide with an elution buffer. The source containing the *mpl* ligand may be plasma where the immobilized receptor is preferably a *mpl*-IgG fusion.

Alternatively, the source containing the *mpl* ligand is recombinant cell culture where the concentration of *mpl* ligand in either the culture medium or in cell lysates is generally higher than in plasma or other natural sources. In this case the above described *mpl*-IgG immunoaffinity method, while still useful, is usually not necessary and more traditional protein purification methods known in the art may be applied.

Briefly, the preferred purification method to provide substantially homogeneous *mpl* ligand comprises: removing particulate debris, either host cells or lysed fragments by, for example, centrifugation or ultrafiltration; optionally, protein may be concentrated with a commercially available protein concentration filter; followed by separating the ligand from other impurities by one or more steps selected from; immunoaffinity, ion-exchange (e.g., DEAE or matrices containing carboxymethyl or sulfopropyl groups), Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toypearl, Butyl Toypearl, Phenyl Toypearl, protein A Sepharose, SDS-PAGE, reverse phase HPLC (e.g., silica gel with appended aliphatic groups) or Sephadex molecular sieve or size exclusion chromatography, and ethanol or ammonium sulfate precipitation. A protease inhibitor such as methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis.

In another preferred embodiment, this invention provides an isolated antibody capable of binding to the *mpl* ligand. A preferred *mpl* ligand isolated antibody is monoclonal (Kohler and Milstein, *Nature*, 256:495-497 [1975]; Campbell, *Laboratory Techniques in Biochemistry and Molecular Biology*, Burdon et al., Eds., Volume 13, Elsevier Science Publishers, Amsterdam [1985]; and Huse et al., *Science*, 246:1275-1281 [1989]). Preferred *mpl* ligand isolated antibody is one that binds

to *mpl* ligand with an affinity of at least about  $10^6$  l/mole. More preferably the antibody binds with an affinity of at least about  $10^7$  l/mole. Most preferably, the antibody is raised against the *mpl* ligand having one of the above described effector functions. The isolated antibody capable of binding to the *mpl* ligand may optionally be fused to a second polypeptide and the antibody or fusion thereof may be used to isolate and purify *mpl* ligand from a source as described above for immobilized *mpl* polypeptide. In a further preferred aspect of this embodiment, the invention provides a method for detecting the *mpl* ligand *in vitro* or *in vivo* comprising contacting the antibody with a sample, especially a serum sample, suspected of containing the ligand and detecting if binding has occurred.

10 In still further preferred embodiments, the invention provides an isolated nucleic acid molecule encoding the *mpl* ligand or fragments thereof, which nucleic acid molecule may be labeled or unlabeled with a detectable moiety, and a nucleic acid molecule having a sequence that is complementary to, or hybridizes under stringent or moderately stringent conditions with, a nucleic acid molecule having a sequence encoding a *mpl* ligand. A preferred *mpl* ligand nucleic acid is RNA or DNA that encodes a biologically active *mpl* ligand sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95% sequence identity with the human *mpl* ligand. More preferred isolated nucleic acid molecules are DNA sequences encoding biologically active *mpl* ligand, selected from: (a) DNA based on the coding region of a mammalian *mpl* ligand gene (e.g., DNA comprising the nucleotide sequence provided in Fig. 1 (SEQ ID NO: 2), or fragments thereof); (b) DNA capable of hybridizing to a DNA of (a) under at least moderately stringent conditions; and (c) DNA that is degenerate to a DNA defined in (a) or (b) which results from degeneracy of the genetic code. It is contemplated that the novel *mpl* ligands described herein may be members of a family of ligands or cytokines having suitable sequence identity that their DNA may hybridize with the DNA of Fig. 1 (SEQ ID NO: 2) (or the complement or fragments thereof) under low to moderate stringency conditions. Thus a further aspect of this invention 15 includes DNA that hybridizes under low to moderate stringency conditions with DNA encoding the *mpl* ligand polypeptides.

20

25

30

35 In a further preferred embodiment of this invention, the nucleic acid molecule is cDNA encoding the *mpl* ligand and further comprises a replicable vector in which the cDNA is operably linked to control sequences recognized by a host transformed with the vector. This aspect further includes host cells transformed with the vector and a method of using the cDNA to effect production of *mpl* ligand, comprising expressing the cDNA encoding the *mpl* ligand in a culture of the transformed host cells and recovering the *mpl* ligand from the host cell culture. The *mpl* ligand prepared in this manner is

preferably substantially homogeneous human *mpl* ligand. A preferred host cell for producing *mpl* ligand is Chinese hamster ovary (CHO) cells.

The invention further includes a preferred method for treating a mammal having an immunological or hematopoietic disorder, especially thrombocytopenia comprising administering a therapeutically effective amount of a *mpl* ligand to the mammal. Optionally, the *mpl* ligand is administered in combination with a cytokine, especially a colony stimulating factor or interleukin. Preferred colony stimulating factors or interleukins include; kit-ligand, LIF, G-CSF, GM-CSF, M-CSF, EPO, IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 or IL-11.

10

### III. Methods of Making

Platelet production has long been thought by some authors to be controlled by multiple lineage specific humoral factors. It has been postulated that two distinct cytokine activities, referred to as megakaryocyte colony-stimulating factor (meg-CSF) and thrombopoietin, regulate megakaryocytopoiesis and thrombopoiesis (Williams *et al.*, *J. Cell Physiol.*, 110:101-104 [1982]; Williams *et al.*, *Blood Cells*, 15:123-133 [1989]; and Gordon *et al.*, *Blood*, 80:302-307 [1992]). According to this hypothesis, meg-CSF stimulates the proliferation of progenitor megakaryocytes while thrombopoietin primarily affects maturation of more differentiated cells and ultimately platelet release. Since the 1960's the induction and appearance of both meg-CSF and thrombopoietin activities in the plasma, serum and urine of animals and humans following thrombocytopenic episodes has been well documented (Odell *et al.*, *Proc. Soc. Exp. Biol. Med.*, 108:428-431 [1961]; Nakeff *et al.*, *Acta Haematol.*, 54:340-344 [1975]; Specter, *Proc. Soc. Exp. Biol.*, 108:146-25 149 [1961]; Schreiner *et al.*, *J. Clin. Invest.*, 49:1709-1713 [1970]; Ebbe, *Blood*, 44:605-608 [1974]; Hoffman *et al.*, *N. Engl. J. Med.*, 305:533 [1981]; Straneva *et al.*, *Exp. Hematol.*, 17:1122-1127 [1988]; Mazur *et al.*, *Exp. Hematol.*, 13:1164 [1985]; Mazur *et al.*, *J. Clin. Invest.*, 68:733-741 [1981]; Sheiner *et al.*, *Blood*, 56:183-188 [1980]; Hill *et al.*, *Exp. Hematol.*, 20:354-360 [1992]; and Hegyi *et al.*, *Int. J. Cell Cloning*, 8:236-244 [1990]). These activities were reported to be lineage specific and distinct from known cytokines (Hill R.J. *et al.*, *Blood* 80:346 (1992); Erickson-Miller C.L. *et al.*, *Brit. J. Haematol.*, 84:197-203 (1993); Straneva J.E. *et al.*, *Exp. Hematol.* 20:4750(1992); and Tsukada J. *et al.*, *Blood* 81:866-867 [1993]). Heretofore, attempts to purify meg-CSF or thrombopoietin from thrombocytopenic plasma or urine have been unsuccessful.

Consistent with the above observations describing thrombocytopenic plasma, we have found that aplastic porcine plasma (APP) obtained from irradiated pigs stimulates human megakaryocytopoiesis *in vitro*. We have found that this stimulatory

activity is abrogated by the soluble extracellular domain of *c-mpl*, confirming APP as a potential source of the putative *mpl* ligand (ML). We have now successfully purified the *mpl* ligand from APP and amino acid sequence information was used to isolate murine, porcine and human ML cDNA. These ML's have sequence homology to erythropoietin and have both meg-CSF and thrombopoietin-like activities.

5        1. Purification and Identification of *mpl* Ligand from Plasma

As set forth above, aplastic plasma from a variety of species has been reported to contain activities that stimulate hematopoiesis *in vitro*, however no hematopoietic 10 stimulatory factor has previously been reported isolated from plasma. One source of aplastic plasma is that obtained from irradiated pigs. This aplastic porcine plasma (APP) stimulates human hematopoiesis *in vitro*. To determine if APP contained the *mpl* ligand, its effect was assayed by measuring  $^3\text{H}$ -thymidine incorporation into Ba/F3 cells transfected with human *mpl* P (Ba/F3-*mpl*) by the procedure shown in 15 Fig. 2. APP stimulated  $^3\text{H}$ -thymidine incorporation into Ba/F3-*mpl* cells but not Ba/F3 control cells (*i.e.*, not transfected with human *mpl* P). Additionally, no such activity was observed in normal porcine plasma. These results indicated that APP contained a factor or factors that transduced a proliferative signal through the *mpl* receptor and therefore might be the natural ligand for this receptor. This was further 20 supported by the finding that treatment of APP with soluble *mpl*-IgG blocked the stimulatory effects of APP on Ba/F3-*mpl* cells.

The activity in APP appeared to be a protein since pronase, DTT, or heat 25 destroy the activity in APP (Fig. 3). The activity was also non-dialyzable. The activity was, however, stable to low pH (pH 2.5 for 2 hrs.) and was shown to bind and elute from several lectin-affinity columns, indicating that it was a glycoprotein. To further elucidate the structure and identity of this activity it was affinity purified from APP using a *mpl*-IgG chimera.

APP was treated according to the protocol set forth in Examples 1 and 2. Briefly, the *mpl* ligand was purified using hydrophobic interaction chromatography 30 (HIC), immobilized dye chromatography, and *mpl*-affinity chromatography. The recovery of activity from each step is shown in Fig. 4 and the fold purification is provided in Table 1. The overall recovery of activity through the *mpl*-affinity column was approximately 10%. The peak activity fraction (F6) from the *mpl*-affinity column has an estimated specific activity of  $9.8 \times 10^6$  units/mg. The overall 35 purification from 5 liters of APP was approximately 4  $\times 10^6$  fold (0.8 units/mg to  $3.3 \times 10^6$  units/mg) with a  $83 \times 10^6$ -fold reduction in protein (250 gms to 3  $\mu\text{g}$ ). We estimated the specific activity of the ligand eluted from the *mpl*-affinity column to be  $\sim 3 \times 10^6$  units/mg..

TABLE 1  
Purification of *mpl* Ligand

Sample	Volume mls	Protein mg/ml	Units/ml	Units	Specific Activity Units/mg	Yield %	Fold Purification
APP	5000	50	40	200,000	0.8	-	1
Phenyl	4700	0.8	40	200,000	50	94	62
Blue-Sep.	640	0.93	400	256,000	430	128	538
<i>mpl</i> ( $\mu$ l) (Fxns 5-7)	12	$5 \times 10^{-4}$	1666	20,000	3,300,000	10	4,100,000

Protein was determined by the Bradford assay. Protein concentration of *mpl*-eluted fractions 5 5-7 are estimates based on staining intensity of a silver stained SDS-gel. One unit is defined as that causing 50% maximal stimulation of Ba/F3-*mpl* cell proliferation.

Analysis of eluted fractions from the *mpl* affinity column by SDS-PAGE (4-10 20%, Novex gel) run under reducing conditions, revealed the presence of several proteins (Fig. 5). Proteins that silver stained with the strongest intensity resolved with apparent Mr of 66,000, 55,000, 30,000, 28,000 and 18,000-22,000. To determine which of these proteins stimulated proliferation of Ba/F3-*mpl* cell cultures, the proteins were eluted from the gel as described in Example 2.

15 The results of this experiment showed that most of the activity eluted from a gel slice that included proteins with Mr 28,000-32,000, with lesser activity eluting in the 18,000-22,000 region of the gel (Fig. 6). The only proteins visible in these regions had Mr of 30,000, 28,000 and 18,000-22,000. To identify and obtain protein sequence for the proteins resolving in this region of the gel (i.e. bands at 30, 20 28 and 18-22 kDa), these three proteins were electroblotted to PVDF and sequenced as described in Example 3. Amino-terminus sequences obtained are provided in Table 2.

**TABLE 2**  
***Mpl Ligand Amino-Terminus Sequences***

30 kDa						
1	5	10	15	20	25	
(S) P A P P A(C)D P R L L N K L L R D D (H/S) V L H (G) R L						(SEQ ID NO: 30)
28 kDa						
1	5	10	15	20	25	
(S) P A P P A X D P R L L N K L L R D D (H) V L (H) G R						(SEQ ID NO: 31)
18-22 kDa						
1	5	10				
X P A P P A X D P R L X (N) (K)						(SEQ ID NO: 32)

Computer-assisted analysis revealed these amino acid sequences to be novel.

5 Because all three sequences were the same, it was believed the 30 kDa, 28 kDa and 18-22 kDa proteins were related and might be different forms of the same novel protein. Furthermore, this protein(s) was a likely candidate as the natural *mpl* ligand because the activity resolved on SDS-PAGE in the same region (28,000-32,000) of a 4-20% gel. In addition, the partially purified ligand migrated with a Mr of 17,000-10 30,000 when subjected to gel filtration chromatography using a Superose 12 (Pharmacia) column. It is believed the different Mr forms of the ligand are a result of proteolysis or glycosylation differences or other post or pre-translational modifications.

As described earlier, antisense human *mpl* RNA abrogated 15 megakaryopoiesis in human bone marrow cultures enriched with CD 34+ progenitor cells without affecting the differentiation of other hematopoietic cell lineages (Methia *et al.*, *supra*). This result suggested that the *mpl* receptor might play a role in the differentiation and proliferation of megakaryocytes *in vitro*. To further elucidate the role of the *mpl* ligand in megakaryopoiesis, the effects of APP and *mpl* 20 ligand depleted APP on *in vitro* human megakaryopoiesis was compared. The effect of APP on human megakaryopoiesis was determined using a modification of the liquid suspension megakaryopoiesis assay described in Example 4. In this assay, human peripheral stem cells (PSC) were treated with APP before and after *mpl*-IgG affinity chromatography. GPIIb/IIIa stimulation of megakaryopoiesis was 25 quantitated with an <sup>125</sup>I-anti-IIb/IIIa antibody (Fig. 7). Shown in Fig. 7, 10% APP caused approximately a 3-fold stimulation while APP depleted of *mpl* ligand had no effect. Significantly, the *mpl* ligand depleted APP did not induce proliferation of the Ba/F3-*mpl* cells.

In another experiment, soluble human *mp1*-IgG added at days 0, 2 and 4 to cultures containing 10% APP neutralized the stimulatory effects of APP on human megakaryocytopoiesis (Fig. 8). These results indicate that the *mp1* ligand plays a role in regulating human megakaryocytopoiesis and therefore may be useful for the treatment of thrombocytopenia.

## 2. Molecular Cloning of the *mp1* Ligand

Based on the amino-terminal amino acid sequence obtained from the 30 kDa, 28 kDa and 18-22 kDa proteins (see Table 2 above), two degenerate oligonucleotide primer pools were designed and used to amplify porcine genomic DNA by PCR. It was reasoned that if the amino-terminal amino acid sequence was encoded by a single exon then the correct PCR product was expected to be 69 bp long. A DNA fragment of this size was found and subcloned into pGEMT. The sequences of the oligonucleotide PCR primers and the three clones obtained are shown in Example 5. The amino acid sequence (PRLLNKLLR [SEQ ID NO: 33]) of the peptide encoded between the PCR primers was identical to that obtained by amino-terminal protein sequencing of the porcine ligand (see residues 9-17 for the 28 and 30 kDa porcine protein sequences above).

A synthetic oligonucleotide based on the sequence of the PCR fragment was used to screen a human genomic DNA library. A 45-mer oligonucleotide, designated pR45, was designed and synthesized based on the sequence of the PCR fragment. This oligonucleotide had the following sequence:

5' GCC-GTG-AAG-GAC-GTG-GTC-GTC-ACG-AAG-CAG-TTT-ATT-TAG-GAG-TCG 3'  
(SEQ ID NO: 34)

This deoxyoligonucleotide was used to screen a human genomic DNA library in  $\lambda$ gem12 under low stringency hybridization and wash conditions according to Example 6. Positive clones were picked, plaque purified and analyzed by restriction mapping and southern blotting. A 390 bp EcoRI-XbaI fragment that hybridized to the 45-mer was subcloned into pBluescript SK-. DNA sequencing of this clone confirmed that DNA encoding the human homolog of the porcine *mp1* ligand had been isolated. The human DNA sequence and deduced amino acid sequence are shown in Fig. 9 (SEQ ID NOS: 3 & 4). The predicted positions of introns in the genomic sequence are also indicated by arrows, and define a putative exon ("exon 3").

Based on the human "exon 3" sequence (Example 6) oligonucleotides corresponding to the 3' and 5' ends of the exon sequence were synthesized. These 2 primers were used in PCR reactions employing as a template cDNA prepared from various human tissues. The expected size of the correct PCR product was 140 bp. After analysis of the PCR products on a 12% polyacrylamide gel, a DNA fragment of the

expected size was detected in cDNA libraries prepared from human adult kidney, 293 fetal kidney cells and cDNA prepared from human fetal liver.

A fetal liver cDNA library ( $7 \times 10^6$  clones) in lambda DR2 was next screened with the same 45-mer oligonucleotide used to screen the human genomic library and 5 the fetal liver cDNA library under low stringency hybridization conditions. Positive clones were picked, plaque purified and the insert size was determined by PCR. One clone with a 1.8 kb insert was selected for further analysis. Using the procedures described in Example 7 the nucleotide and deduced amino acid sequence of the human *mpl* ligand (hML) were obtained. These sequences are presented in Fig. 1 (SEQ ID 10 NOS: 1 & 2).

### 3. Structure of the Human *mpl* Ligand (hML)

The human *mpl* ligand (hML) cDNA sequence (Fig. 1 [SEQ ID NO: 2]) comprises 1774 nucleotides followed by a poly(A) tail. It contains 215 nucleotides of 15 5' untranslated sequence and a 3' untranslated region of 498 nucleotides. The presumed initiation codon at nucleotide position (216-218) is within a consensus sequence favorable for eukaryotic translation initiation. The open reading frame is 1059 nucleotides long and encodes a 353 amino acid residue polypeptide, beginning at nucleotide position 220. The N-terminus of the predicted amino acid sequence is 20 highly hydrophobic and probably corresponds to a signal peptide. Computer analysis of the predicted amino acid sequence (von Heijne *et al.*, *Eur. J. Biochem.*, 133:17-21 [1983]) indicates a potential cleavage site for signal peptidase between residues 21 and 22. Cleavage at that position would generate a mature polypeptide of 332 amino acid residues beginning with the amino-terminal sequence obtained from *mpl* ligand 25 purified from porcine plasma. The predicted non-glycosylated molecular weight of the 332 amino acid residue ligand is about 38 kDa. There are 6 potential N-glycosylation sites and 4 cysteine residues.

Comparison of the *mpl* ligand sequence with the Genbank sequence database 30 revealed 23% identity between the amino terminal 153 residues of mature human *mpl* ligand and human erythropoietin (Fig. 10 [SEQ ID NOS: 6 & 7]). When conservative substitutions are taken into account, this region of hML shows 50% similarity to human erythropoietin (hEPO). Both hEPO and the hML contain four cysteines. Three of the 4 cysteines are conserved in hML, including the first and last cysteines. Site-directed mutagenesis experiments have shown that the first and last cysteines of 35 erythropoietin form a disulfide bond that is required for function (Wang, F.F. *et al.*, *Endocrinology* 116:2286-2292 [1983]). By analogy, the first and last cysteines of hML may also form a critical disulfide bond. None of the glycosylation sites are

conserved in hML. All potential hML N-linked glycosylation sites are located in the carboxy-terminal half of the hML polypeptide.

Similar to hEPO, the hML mRNA does not contain the consensus polyadenylation sequence AAUAAA, nor the regulatory element AUUUA that is present in 3' untranslated regions of many cytokines and is thought to influence mRNA stability (Shaw *et al.*, *Cell*, 46:659-667 [1986]). Northern blot analysis reveals low levels of a single 1.8 kb hML RNA transcript in both fetal and adult liver. After longer exposure, a weaker band of the same size could be detected in adult kidney. By comparison, human erythropoietin is expressed in fetal liver and, in response to hypoxia, the adult kidney and liver (Jacobs *et al.*, *Nature*, 313:804-809 [1985] and Bondurant *et al.*, *Molec. Cell. Biol.*, 6:2731-2733 [1986]).

The importance of the C-terminal region of the hML remains to be elucidated. Based on the presence of the six potential sites for N-linked glycosylation and the ability of the ligand to bind lectin-affinity columns, this region of the hML is likely glycosylated. In some gel elution experiments, we observed activity resolving with a Mr around 60,000 which may represent the full length, glycosylated molecule. The C-terminal region may therefore act to stabilize and increase the half-life of circulating hML. In the case of erythropoietin, the non-glycosylated form has full *in vitro* biological activity, but has a significantly reduced plasma half-life relative to glycosylated erythropoietin (Takeuchi *et al.*, *J. Biol. Chem.*, 265:12127-12130 [1990]; Narhi *et al.*, *J. Biol. Chem.*, 266:23022-23026 [1991] and Spivack *et al.*, *Blood*, 7:90-99 [1989]). The C-terminal domain of hML contains two di-basic amino acid sequences [Arg-Arg motifs at positions 153-154 and 245-246] that could serve as potential processing sites. Cleavage at these sites may be responsible for generating the 30, 28 and 18-22 kDa forms of the ML isolated from APP. Significantly, the Arg<sub>153</sub>-Arg<sub>154</sub> sequence occurs immediately following the erythropoietin-like domain of the ML. These observations indicate that full length ML may represent a precursor protein that undergoes limited proteolysis to generate the mature ligand.

30

#### 4. Isoforms and Variants of the Human *mpl* Ligand

Isoforms or alternatively spliced forms of human *mpl* ligand were detected by PCR in human adult liver. Briefly, primers were synthesized corresponding to each end as well as selected internal regions of the coding sequence of hML. These primers 35 were used in RT-PCR to amplify human adult liver RNA as described in Example 10. In addition to the full length form, designated hML, three other forms, designated hML2, hML3 and hML4, were observed or deduced. The mature deduced amino acid sequences of all four isoforms is presented in Fig. 11 (SEQ ID NOS: 6, 8, 9 & 10).

hML3 has a 116 nucleotide deletion a position 700 which results in both an amino acid deletion and a frameshift. The cDNA now encodes a mature polypeptide that is 265 amino acid long and diverges from the hML sequence at amino acid residue 139. Finally, hML4 has both a 12 nucleotide deletion following nucleotide position 618

5 (also found in the mouse and the pig sequences [see below]) and the 116 bp deletion found in hML3. Although no clones with only the 12 bp deletion (following nucleotide 619) have been isolated in the human (designated hML2), this form is likely to exist because such a isoform has been identified in both the mouse and pig (see below), and because it has been identified in conjunction with the 116 nucleotide deletion in hML4.

10 Both a substitutional variant of hML in which the dibasic Arg153-Arg154 sequence was replaced with two alanine residues and a "EPO-domain" truncated form of hML were constructed to determine whether the full length ML was necessary for biological activity. The Arg153-Arg154 dibasic sequence substitutional variant, referred to as hML(R153A, R154A), was constructed using PCR as described in

15 Example 10. The "EPO-domain" truncated form, hML153, was also made using PCR by introducing a stop codon following Arg153.

20

5. Expression of Recombinant Human *mpl* Ligand (rhML) In  
Transiently Transfected Human Embryonic Kidney (293)  
Cells

To confirm that the cloned human cDNA encoded a ligand for *mpl*, the ligand was expressed in mammalian 293 cells under the control of the cytomegalovirus immediate early promoter using the expression vectors pRK5-hML or pRK5-hML153. Supernatants from transiently transfected human embryonic kidney 293 cells were

25 found to stimulate  $^3$ H-thymidine incorporation in Ba/F3-*mpl* cells, but not in parental Ba/F3 cells (Fig. 12A). Media from the 293 cells transfected with the pRK vector alone did not contain this activity. Addition of *mpl*-IgG to the media abolished the stimulation (data not shown). These results show that the cloned cDNA encodes a functional human ML (hML).

30 To determine if the "EPO-domain" alone could bind and activate *mpl*, the truncated form of hML, rhML153, was expressed in 293 cells. Supernatants from transfected cells were found to have activity similar to that present in supernatants from cells expressing the full length hML (Fig. 12A), indicating that the C-terminal domain of ML is not required for binding and activation of c-*mpl*.

35

6. *mp1 Ligand Stimulates Megakaryocytopoiesis and Thrombopoiesis*

Both the full length rhML and the truncated rhML<sub>153</sub> forms of recombinant hML stimulated human megakaryocytopoiesis *in vitro* (Fig. 12B). This effect was observed in the absence of other exogenously added hematopoietic growth factors. With the exception of IL-3, the ML was the only hematopoietic growth factor tested that exhibited this activity. IL-11, IL-6, IL-1, erythropoletin, G-CSF, IL-9, LIF, kit ligand (KL), M-CSF, OSM and GM-CSF had no effect on megakaryocytopoiesis when tested separately in our assay (data not shown). This result demonstrates that the ML has megakaryocyte-stimulating activity, and indicates a role for ML in regulating megakaryocytopoiesis.

Thrombopoietic activities present in plasma of thrombocytopenic animals have been shown to stimulate platelet production in a mouse rebound thrombocytosis assay (McDonald, *Proc. Soc. Exp. Biol. Med.*, 14:1006-1001 [1973] and McDonald *et al.*, 15 *Scand. J. Haematol.*, 16:326-334 [1976]). In this model mice are made acutely thrombocytopenic using specific antiplatelet serum, resulting in a predictable rebound thrombocytosis. Such immuno-thrombocytemic mice are more responsive to exogenous thrombopoietin-like activities than are normal mice (McDonald, *Proc. Soc. Exp. Biol. Med.*, 14:1006-1001 [1973]), just as exhypoxic mice are more sensitive to erythropoietin than normal are mice (McDonald, *et al.*, *J. Lab. Clin. Med.*, 77:134-143 [1971]). To determine whether the rML stimulates platelet production *in vivo*, mice in rebound thrombocytosis were injected with partially purified rhML. Platelet counts and incorporation of <sup>35</sup>S into platelets were then quantitated. Injection of mice with 64,000 or 32,000 units of rML significantly increased platelet production, as evidenced by a ~20% increase in platelet counts ( $p=0.0005$  and  $0.0001$ , respectively) and a ~40% increase in <sup>35</sup>S incorporation into platelets ( $p=0.003$ ) in the treated mice versus control mice injected with excipient alone (Fig. 12C). This level of stimulation is comparable to that which we have observed with IL-6 in this model (data not shown). Treatment with 16,000 units of rML did not significantly stimulate platelet production. These results indicate that ML stimulates platelet production in a dose-dependent manner and therefore possesses thrombopoietin-like activity.

293 cells were also transfected with the other hML isoform constructs described above and the supernatants were assayed using the Ba/F3-*mp1* proliferation assay (see Fig. 13). hML2 and hML3 showed no detectable activity in this assay, however the activity of hML(R153A, R154A) was similar to hML and hML<sub>153</sub> indicating that processing at the Arg153-Arg154 di-basic site is neither required for nor detrimental to activity.

### 7. Megakary cytopoiesis and the *mpl* Ligand

It has been proposed that megakaryocytopoiesis is regulated at multiple cellular levels (Williams *et al.*, *J. Cell. Physiol.*, 110:101-104 [1982] and Williams 5 *et al.*, *Blood Cells*, 15:123-133 [1989]). This is based largely on the observation that certain hematopoietic growth factors stimulate proliferation of megakaryocyte progenitors while others appear to primarily affect maturation. The results presented here suggest that the ML acts both as a proliferative and maturation factor. That ML stimulates proliferation of megakaryocyte progenitors is supported by several lines of 10 evidence. First, APP stimulates both proliferation and maturation of human megakaryocytes *in vitro*, and this stimulation is completely inhibited by *mpl*-IgG (Figs. 7 and 8). Furthermore, the inhibition of megakaryocyte colony formation by c-*mpl* antisense oligonucleotides (Methia *et al.*, *Blood*, 82:1395-1401 [1993]) and the finding that c-*mpl* can transduce a proliferative signal in cells into which it is 15 transfected (Skoda *et al.*, *EMBO*, 12:2645-2653 [1993] and Vigon *et al.*, *Oncogene*, 8:2607-2615 [1993]) also indicate that ML stimulates proliferation. The apparent expression of c-*mpl* during all stages of megakaryocyte differentiation (Methia *et al.*, *Blood*, 82:1395-1401 [1993]) and the ability of recombinant ML to rapidly 20 stimulate platelet production *in vivo* indicate that ML also affects maturation. The availability of recombinant ML makes possible a careful evaluation of its role in regulating megakaryocytopoiesis and thrombopoiesis as well as its potential to influence other hematopoietic lineages.

### 8. Isolation of the Human *mpl* Ligand (TPO) Gene

25 Human genomic DNA clones of the TPO gene were isolated by screening a human genomic library in  $\lambda$ -Gem12 with pR45, under low stringency conditions or under high stringency conditions with a fragment corresponding to the 3' half of human cDNA coding for the *mpl* ligand. Two overlapping lambda clones spanning 35 kb were isolated. Two overlapping fragments (BamH1 and EcoRI) containing the entire TPO 30 gene were subcloned and sequenced (see Figs. 14A, 14B and 14C).

The structure of the human gene is composed of 6 exons within 7 kb of genomic DNA. The boundaries of all exon/intron junctions are consistent with the consensus motif established for mammalian genes (Shapiro, M. B., *et al.*, *Nucl. Acids Res.* 15:7155 [1987]). Exon 1 and exon 2 contain 5' untranslated sequence and the initial 35 four amino acids of the signal peptide. The remainder of the secretory signal and the first 26 amino acids of the mature protein are encoded within exon 3. The entire carboxyl domain and 3' untranslated as well as ~50 amino acids of the erythropoietin-

like domain are encoded within exon 6. The four amino acids involved in the deletion observed within hML-2 (hTPO-2) are encoded at the 5' end of exon 6.

Analysis of human genomic DNA by Southern blot indicated the gene for TPO is present in a single copy. The chromosomal location of the gene was determined by 5 fluorescent *in situ* hybridization (FISH) which mapped to chromosome 3q27-28.

#### 9. Expression and Purification of TPO from 293 Cells

Preparation and purification of ML or TPO from 293 cells is described in detail in Example 19. Briefly, cDNA corresponding to the TPO entire open reading frame 10 was obtained by PCR using pRK5-hmp1 I. The PCR product was purified and cloned between the restriction sites Clal and XbaI of the plasmid pRK5tkneo (a pRK5 derived vector modified to express a neomycin resistance gene under the control of the thymidine kinase promoter) to obtain the vector pRK5tkneo.ORF(a vector coding for the entire open reading frame).

15 A second vector coding for the EPO homologous domain was generated the same but using different PCR primers to obtain the final construct called pRK5-tkneoEPO-D.

20 These two constructs were transfected into Human Embryonic Kidney cells by the CaPO<sub>4</sub> method and neomycin resistant clones were selected and allowed to grow to confluence. Expression of ML153 or ML332 in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay.

25 Purification of rhML332 was conducted as described in Example 19. Briefly, 293-rhML332 conditioned media was applied to a Blue-Sepharose (Pharmacia) column that was subsequently washed with a buffer containing 2M urea. The column was eluted with a buffer containing 2M urea and 1M NaCl. The Blue-Sepharose elution pool was then directly applied to a WGA-Sepharose column, washed with 10 column volumes of buffer containing 2M urea and 1 M NaCl and eluted with the same buffer containing 0.5M N-acetyl-D-glucosamine. The WGA-Sepharose eluate was applied to a C4-HPLC column (Synchrom, Inc.) and eluted with a discontinuous 30 propanol gradient. By SDS-PAGE the purified 293-rhML332 migrates as a broad band in the 68-80 kDa region of the gel (see Fig. 15).

35 Purification of rhML153 was also conducted as described in Example 19. Briefly, 293-rhML153 conditioned media was resolved on Blue-Sepharose as described for rhML332. The Blue Sepharose eluate was applied directly to a *mpl*-affinity column as described above. RhML153 eluted from the *mpl*-affinity column was purified to homogeneity using a C4-HPLC column run under the same conditions used for rhML332. By SDS-PAGE the purified rhML153 resolves into 2 major and 2 minor bands with Mr of ~18,000-22,000 (see Fig. 15).

#### 10. The Murine *mp1* Ligand

A DNA fragment corresponding to the coding region of the human *mp1* ligand was obtained by PCR, gel purified and labeled in the presence of  $^{32}\text{P}$ -dATP and  $^{32}\text{P}$ -dCTP.

5 This probe was used to screen  $10^6$  clones of a mouse liver cDNA library in  $\lambda$ GT10. A murine clone (Fig. 16 [SEQ ID NOS: 12 & 13]) containing a 1443 base pair insert was isolated and sequenced. The presumed initiation codon at nucleotide position 138-141 was within a consensus sequence favorable for eukaryotic translation initiation (Kozak, M. *J. Cell. Biol.*, 108:229-241 [1989]). This sequence defines an open

10 reading frame of 1056 nucleotides, which predicts a primary translation product of 352 amino acids. Flanking this open reading frame are 137 nucleotides of 5' and 247 nucleotides of 3' untranslated sequence. There is no poly(A) tail following the 3' untranslated region indicating that the clone is probably not complete. The N-terminus of the predicted amino acid sequence is highly hydrophobic and probably represents a

15 signal peptide. Computer analysis (von Heijne, G. *Eur. J. Biochem.* 133:17-21 [1983]) indicated a potential cleavage site for signal peptidase between residues 21 and 22. Cleavage at that position would generate a mature polypeptide of 331 amino acids (35 kDa) identified as mML331 (or mML2 for reasons described below). The sequence contains 4 cysteines, all conserved in the human sequence, and seven

20 potential N-glycosylation sites, 5 of which are conserved in the human sequence. Again, as with hML, all seven potential N-glycosylation sites are located in the C-terminal half of the protein.

When compared with the human ML, considerable identity for both nucleotide and deduced amino acid sequences were observed in the "EPO-domains" of these ML's.

25 However, when deduced amino acid sequences of human and mouse ML's were aligned, the mouse sequence appeared to have a tetrapeptide deletion between residues 111-114 corresponding to the 12 nucleotide deletion following nucleotide position 618 seen in both the human (see above) and pig (see below) cDNA's. Accordingly, additional clones were examined to detect possible murine ML isoforms. One clone encoded a 335

30 amino acid deduced sequence polypeptide containing the "missing" tetrapeptide LPLQ. This form is believed to be the full length murine ML and is referred to as mML or mML335. The nucleotide and deduced amino acid sequence for mML are provided in Fig. 17 (SEQ ID NOS: 14 & 15). This cDNA clone consists of 1443 base pairs followed by a poly(A) tail. It possesses an open reading frame of 1068 bp flanked by

35 134 bases of 5' and 241 bases of 3' untranslated sequence. The presumed initiation codon lies at nucleotide position 138-140. The open reading frame encodes a predicted protein of 356 amino acids, the first 21 of which are highly hydrophobic and likely function as a secretion signal.

Finally, a third murine clone was isolated, sequenced and was found to contained the 116 nucleotide deletion corresponding to hML3. This murine isoform is therefore denominated mML3. Comparison of the deduced amino acid sequences of these two isoforms is shown in Fig. 18 (SEQ ID NOS: 9 & 16).

5 The overall amino acid sequence identity between human and mouse ML (Fig. 19 [SEQ ID NOS: 6 & 17]) is 72% but this homology is not evenly distributed. The region defined as the "EPO-domain" (amino acids 1-153 for the human sequence and 1-149 for the mouse) is better conserved (86% homology) than the carboxy-terminal region of the protein (62% homology). This may further indicate that only  
10 the "EPO-domain" is important for the biological activity of the protein. Interestingly, of the two di-basic amino acid motifs found in hML, only the di-basic motif immediately following the "EPO-domain" (residue position 153-154) in the human sequence is present in the murine sequence. This is consistent with the possibility that the full length ML may represent a precursor protein that undergoes  
15 limited proteolysis to generate the mature ligand. Alternatively, proteolysis between Arg153-Arg154 may facilitate hML clearance

An expression vector containing the entire coding sequence of mML was transiently transfected into 293 cells as described in Example 1. Conditioned media from these cells stimulated  $^3$ H-thymidine incorporation into Ba/F3 cells expressing 20 either murine or human *mp1* but had no effect on the parental (*mp1*-less) cell line. This indicates that the cloned murine ML cDNA encodes a functional ligand that is able to activate both the murine and human ML receptor (*mp1*).

#### 11. The Porcine *mp1* Ligand

25 Porcine ML (pML) cDNA was isolated by RACE PCR as described in Example 13. A PCR cDNA product of 1342 bp was found in kidney and subcloned. Several clones were sequenced and found to encode a pig *mp1* ligand of 332 amino acid residues referred to as pML (or pML332) having the nucleotide and deduced amino acid sequence shown in Fig. 20 (SEQ ID NOS: 18 & 19).

30 Again, a second form, designated pML2, encoding a protein with a 4 amino acid residue deletion (228 amino acid residues) was identified (see Fig. 21 [SEQ ID NO: 21]). Comparison of pML and pML2 amino acid sequences shows the latter form is identical except that the tetrapeptide QLPP corresponding to residues 111-114 inclusive have been deleted (see Fig. 22 [SEQ ID NOS: 18 & 21]). The four amino 35 acid deletions observed in both murine and porcine ML cDNA occur at precisely the same position within the predicted proteins.

Comparison of the predicted amino acid sequences of the mature ML from human, mouse, and pig (Fig. 19 [SEQ ID NOS: 6, 17 & 18]) indicates that overall

sequence identity is 72 percent between mouse and human, 68 percent between mouse and pig and 73 percent between pig and human. The homology is substantially greater in the amino-terminal half of the ML (EPO homologous domain). This domain is 80 to 84 percent identical between any two species whereas the carboxy-terminal half (carbohydrate domain) is only 57 to 67 percent identical. A di-basic amino acid motif that could represent a protease cleavage site is present at the carboxyl end of the erythropoietin homology domain. This motif is conserved between the three species at this position (Fig. 19 [SEQ ID NOS: 6, 17 & 18]). A second di-basic site present at position 245 and 246 in the human sequence is not present in the mouse or pig sequences. The murine and the pig ML sequence contain 4 cysteines, all conserved in the human sequence. There are seven potential N-glycosylation sites within the mouse ligand and six within the porcine ML, 5 of which are conserved within the human sequence. Again, all the potential N-glycosylation sites are located in the C-terminal half of the protein.

15

#### 12. Expression and Purification of TPO from Chinese Hamster Ovary (CHO) Cells

The expression vectors used to transfect CHO cells are designated: pSVI5.ID.LL.MLORF (full length or TPO332), and pSVI5.ID.LL.MLEPO-D (truncated or TPO153). The pertinent features of these plasmids are presented in Fig. 23 and 24.

The transfection procedures are described in Example 20. Briefly, cDNA corresponding to the entire open reading frame of TPO was obtained by PCR. The PCR product was purified and cloned between two restriction sites (Cial and SalI) of the plasmid pSVI5.ID.LL to obtain the vector pSVI5.ID.LL.MLORF. A second construct corresponding to the EPO homologous domain was generated the same way but using a different reverse primer(EPOD.SalI). The final construct for the vector coding for the EPO homologous domain of TPO is called pSVI5.ID.LL.MLEPO-D.

These two constructs were linearized with NotI and transfected into Chinese Hamster Ovary Cells (CHO-DP12 cells, EP 307,247 published 15 March 1989) by electroporation.  $10^7$  cells were electroporated in a BRL electroporation apparatus (350 Volts, 330 mF, low capacitance) in the presence of 10, 25 or 50 ng of DNA as described (Andreasen, G.L. *J. Tissue Cult. Meth.* 15,56 [1993]). The day following transfection, cells were split in DHFR selective media (High glucose DMEM-F12 50:50 without glycine, 2mM glutamine, 2-5% dialyzed fetal calf serum). 10 to 15 days later individual colonies were transferred to 96 well plates and allowed to grow to confluence. Expression of ML153 or ML332 in the conditioned media from these clones was assessed using the Ba/F3-mp1 proliferation assay (described in Example 1).

The process for purifying and isolating TPO from harvested CHO cell culture fluid is described in **Example 20**. Briefly, harvested cell culture fluid (HCCF) is applied to a Blue Sepharose column (Pharmacia) at a ratio of approximately 100L of HCCF per liter of resin. The column is then washed with 3 to 5 column volumes of buffer followed by 3 to 5 column volumes of a buffer containing 2.0M urea. TPO is then eluted with 3 to 5 column volumes of buffer containing both 2.0M urea and 1.0M NaCl.

5 The Blue Sepharose eluate pool containing TPO is then applied to a Wheat Germ Lectin Sepharose column (Pharmacia) equilibrated in the Blue Sepharose eluting buffer at a ratio of from 8 to 16 ml of Blue Sepharose eluate per ml of resin. The 10 column is then washed with 2 to 3 column volumes of equilibration buffer. TPO is then eluted with 2 to 5 column volumes of a buffer containing 2.0M urea and 0.5M N-acetyl-D-glucosamine.

15 The Wheat Germ Lectin eluate containing TPO is then acidified and C<sub>12</sub>E<sub>8</sub> is added to a final concentration of 0.04%. The resulting pool is applied to a C4 reversed phase column equilibrated in 0.1% TFA, 0.04% C<sub>12</sub>E<sub>8</sub> at a load of approximately 0.2 to 0.5 mg protein per ml of resin.

20 The protein is eluted in a two phase linear gradient of acetonitrile containing 0.1% TFA and 0.04% C<sub>12</sub>E<sub>8</sub> and a pool is made on the basis of SDS-PAGE.

25 The C4 Pool is then diluted and diafiltrated versus approximately 6 volumes of buffer on an Amicon YM or like ultrafiltration membrane having a 10,000 to 30,000 Dalton molecular weight cut-off. The resulting diafiltrate may be then directly processed or further concentrated by ultrafiltration. The diafiltrate/concentrate is usually adjusted to a final concentration of 0.01% Tween-80.

30 All or a portion of the diafiltrate/concentrate equivalent to 2 to 5% of the calculated column volume is then applied to a Sephadryl S-300 HR column (Pharmacia) equilibrated in a buffer containing 0.01% Tween-80 and chromatographed. The TPO containing fractions which are free of aggregate and proteolytic degradation products are then pooled on the basis of SDS-PAGE. The resulting pool is filtered and stored at 2-8°C.

**13. Methods for Transforming and Inducing TPO Synthesis In a Microorganism and Isolating, Purifying and Refolding TPO Made Therein**

35 Construction of *E. coli* TPO expression vectors is described in detail in **Example 21**. Briefly, plasmids pMP21, pMP151, pMP41, pMP57 and pMP202 were all designed to express the first 155 amino acids of TPO downstream of a small leader which varies among the different constructs. The leaders provide primarily for

high level translation initiation and rapid purification. The plasmids pMP210-1, -T8, -21, -22, -24, -25 are designed to express the first 153 amino acids of TPO downstream of an initiation methionine and differ only in the codon usage for the first 6 amino acids of TPO, while the plasmid pMP251 is a derivative of pMP210-1 in which the carboxy-terminal end of TPO is extended by two amino acids. All of the above plasmids will produce high levels of intracellular expression of TPO in *E. coli* upon induction of the tryptophan promoter (Yansura, D. G. et al. *Methods in Enzymology* ( Goeddel, D. V., Ed.) 185:54-60, Academic Press, San Diego [1990]). The plasmids pMP1 and pMP172 are intermediates in the construction of the above TPO intracellular expression plasmids.

The above TPO expression plasmids were used to transform the *E. coli* using the CaCl<sub>2</sub> heat shock method (Mandel, M. et al. *J. Mol. Biol.*, 53:159-162, [1970]) and other procedures described in Example 21. Briefly, the transformed cells were grown first at 37°C until the optical density (600nm) of the culture reached approximately 2-3. The culture was then diluted and, after growth with aeration, acid was added. The culture was then allowed to continue growing with aeration for another 15 hours after which time the cells were harvested by centrifugation.

The Isolation, Purification and Refolding procedures given below for production of biologically active, refolded human TPO or fragments thereof is described in Examples 22 and 23 can be applied for the recovery of any TPO variant including N and C terminal extended forms. Other procedures suitable for refolding recombinant or synthetic TPO can be found in the following patents; Builder et al., U.S. Patent 4,511,502; Jones et al., U.S. Patent 4,512,922; Olson U.S. Patent 4,518,526 and Builder et al., U.S. Patent 4,620,948; for a general description of the recovery and refolding process for a variety of recombinant proteins expressed in an insoluble form in *E. coli*.

#### A. Recovery of non-soluble TPO

A microorganism such as *E. coli* expressing TPO encoded by any suitable plasmid is fermented under conditions in which TPO is deposited in insoluble "refractile bodies". Optionally, cells are first washed in a cell disruption buffer. Typically, about 100g of cells are resuspended in about 10 volumes of a cell disruption buffer (e.g. 10 mM Tris, 5 mM EDTA, pH 8) with, for example, a Polytron homogenizer and the cells centrifuged at 5000 x g for 30 minutes. Cells are then lysed using any conventional technique such as tonic shock, sonication, pressure cycling, chemical or enzymatic methods. For example, the washed cell pellet above may be resuspended in another 10 volumes of a cell disruption buffer with a homogenizer and the cell suspension is passed through an LH Cell Disrupter (LH Inceltech, Inc.) or through a Microfluidizer (Microfluidics International) according to the manufacturers'

Instructions. The particulate matter containing TPO is then separated from the liquid phase and optionally washed with any suitable liquid. For example, a suspension of cell lysate may be centrifuged at 5,000 X g for 30 minutes, resuspended and optionally centrifuged a second time to make a washed refractile body pellet. The washed pellet 5 may be used immediately or optionally stored frozen (at e.g. -70°C).

*B. Solubilization and Purification of Monomeric TPO*

Insoluble TPO in the refractile body pellet is then solubilized with a solubilizing buffer. The solubilizing buffer contains a chaotropic agent and is usually buffered at a basic pH and contains a reducing agent to improve the yield of monomeric TPO. 10 Representative chaotropic agents include urea, guanidine-HCl, and sodium thiocyanate. A preferred chaotropic agent is guanidine-HCl. The concentration of chaotropic agent is usually 4-9M, preferably 6-8M. The pH of the solubilizing buffer is maintained by any suitable buffer in a pH range of from about 7.5-9.5, preferably 8.0-9.0 and most preferably 8.0. Preferably the solubilizing buffer also contains a reducing agent to aid formation of the monomeric form of TPO. Suitable reducing agents include organic compounds containing a free thiol (RSH). Representative reducing agents include dithiothreitol (DTT), dithioerythritol (DTE), mercaptoethanol, glutathione (GSH), cysteamine and cysteine. A preferred reducing agent is dithiothreitol (DTT). 15 Optionally, the solubilizing buffer may contain a mild oxidizing agent (e.g. molecular oxygen) and a sulfite salt to form monomeric TPO via sulfitolysis. In this embodiment, the resulting TPO-S-sulfonate is later refolded in the presence of the redox buffer (e.g. GSH/GSSG) to form the properly folded TPO.

The TPO protein is usually further purified using, for example, centrifugation, gel filtration chromatography and reversed phase column chromatography.

25 By way of illustration, the following procedure has produced suitable yields of monomeric TPO. The refractile body pellet is resuspended in about 5 volumes by weight of the solubilizing buffer (20 mM Tris, pH 8, with 6-8 M guanidine and 25 mM DTT) and stirred for 1-3 hr., or overnight, at 4°C to effect solubilization of the TPO protein. High concentrations of urea (6-8M) are also useful but generally result 30 in somewhat lower yields compared to guanidine. After solubilization, the solution is centrifuged at 30,000 x g for 30 min. to produce a clear supernatant containing denatured, monomeric TPO protein. The supernatant is then chromatographed on a Superdex 200 gel filtration column (Pharmacia, 2.6 x 60 cm) at a flow rate of 2 ml/min. and the protein eluted with 20 mM Na phosphate, pH 6.0, with 10 mM DTT. 35 Fractions containing monomeric, denatured TPO protein eluting between 160 and 200 ml are pooled. The TPO protein is further purified on a semi-preparative C4 reversed phase column (2 x 20 cm VYDAC). The sample is applied at 5 ml/min. to a column equilibrated in 0.1% TFA (trifluoroacetic acid) with 30% acetonitrile. The protein is

luted with a linear gradient of acetonitrile (30-60% in 60 min.). The purified reduced protein elutes at approximately 50% acetonitrile. This material is used for refolding to obtain biologically active TPO variant.

**C Refolding TPO to Generate the Biologically Active Form**

Following solubilization and further purification of TPO, the biologically active form is obtained by refolding the denatured monomeric TPO in a redox buffer. Because of the high potency of TPO (half maximal stimulation in the Ba/F3 assay is achieved at approximately 3 pg/ml), it is possible to obtain biologically active material utilizing many different buffer, detergent and redox conditions. However, under most conditions only a small amount of properly folded material (<10%) is obtained. For commercial manufacturing processes, it is desirable to have refolding yields at least 10%, more preferably 30-50% and most preferably >50%. Many different detergents including Triton X-100, dodecyl-beta-maltoside, CHAPS, CHAPSO, SDS, sarkosyl, Tween 20 and Tween 80, Zwittergent 3-14 and others were found suitable for producing at least some properly folded material. Of these however, the most preferred detergents were those of the CHAPS family (CHAPS and CHAPSO) which were found to work best in the refolding reaction and to limit protein aggregation and improper disulfide formation. Levels of CHAPS greater than about 1% were most preferred. Sodium chloride was required for the best yields, with the optimal levels between 0.1 M and 0.5M. The presence of EDTA (1-5 mM) in the redox buffer was preferred to limit the amount of metal-catalyzed oxidation (and aggregation) which was observed with some preparations. Glycerol concentrations of greater than 15% produced the optimal refolding conditions. For maximum yields, it was essential to have a redox pair in the redox buffer consisting of both an oxidized and reduced organic thiol (RSH). Suitable redox pairs include mercaptoethanol, glutathione (GSH), cysteamine, cysteine and their corresponding oxidized forms. Preferred redox pairs were glutathione(GSH):oxidized glutathione(GSSG) or cysteine:cystine. The most preferred redox pair was glutathione(GSH):oxidized glutathione(GSSG). Generally higher yields were observed when the mole ratio of oxidized member of the redox pair was equal to or in excess over the reduced member of the redox pair. pH values between 7.5 and about 9 were optimal for refolding of these TPO variants. Organic solvents (e.g. ethanol, acetonitrile, methanol) were tolerated at concentrations of 10-15% or lower. Higher levels of organic solvents increased the amount of improperly folded forms. Tris and phosphate buffers were generally useful. Incubation at 4 °C also produced higher levels of properly folded TPO.

Refolding yields of 40-60% (based on the amount of reduced and denatured TPO used in the refolding reaction) are typical for preparations of TPO that have been purified through the first C4 step. Active material can be obtained when less pure

preparations (e.g. directly after the Superdex 200 column or after the initial refractile body extraction) although the yields are less due to extensive precipitation and interference of non-TPO proteins during the TPO refolding process.

Since TPO contains 4 cysteine residues, it is possible to generate three different disulfide versions of this protein:

version 1: disulfides between cysteine residues 1-4 and 2-3

version 2: disulfides between cysteine residues 1-2 and 3-4

version 3: disulfides between cysteine residues 1-3 and 2-4.

During the initial exploration in determining refolding conditions, several different peaks containing the TPO protein were separated by C4 reversed phase chromatography. Only one of these peaks had significant biological activity as determined using the Ba/F3 assay. Subsequently, the refolding conditions were optimized to yield preferentially that version. Under these conditions, the misfolded versions were less than 10-20% of the total monomeric TPO obtained from the solubilizing step.

The disulfide pattern for the biologically active TPO has been determined to be 1-4 and 2-3 by mass spectrometry and protein sequencing, where the cysteines are numbered sequentially from the amino-terminus. This cysteine cross-linking pattern is consistent with the known disulfide bonding pattern of the related molecule erythropoietin.

#### *D. Biological Activity of Recombinant, Refolded TPO*

Refolded and purified TPO has activity in both *in vitro* and *in vivo* assays. For example, in the Ba/F3 assay, half-maximal stimulation of thymidine incorporation into the Ba/F3 cells for TPO (Met<sup>-1</sup> 1-153) was achieved at 3.3 pg/ml (0.3 pM). In the *mpl* receptor-based ELISA, half-maximal activity occurred at 1.9 ng/ml (120 pM). In normal and myelosuppressed animals produced by near-lethal X-radiation, refolded TPO (Met<sup>-1</sup> 1-153) was highly potent (activity was seen at doses as low as 30 ng/mouse) to stimulate the production of new platelets. Similar biological activity was observed for other forms of TPO refolded in accordance with the above described procedures (see Figs. 25, 26 and 28).

#### **14. Methods for Measurement of Thrombopoietic Activity**

Thrombopoietic activity may be measured in various assays including the Ba/F3 *mpl* ligand assay described in Example 1, an *in vivo* mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured by an anti-platelet immunoassay (anti-GPIIbIIIa) for a human leukemia megakaryoblastic cell line (CMK) (see Sato *et al.*, *Brit. J. Haematol.*, 72:184-190 [1989]) (see also the liquid suspension megakaryocytopoiesis assay described in Example 4), and

Induction of polyploidization in a megakaryoblastic cell line (DAMI) (see Ogura *et al.*, *Blood*, 72(1):49-60 [1988]). Maturation of megakaryocytes from immature, largely non-DNA synthesizing cells, to morphologically identifiable megakaryocytes involves a process that includes appearance of cytoplasmic organelles, acquisition of membrane antigens (GPIIbIII<sub>a</sub>), endoreplication and release of platelets as described in the background. A lineage specific promoter (*i.e.*, the *mpl* ligand) of megakaryocyte maturation would be expected to induce at least some of these changes in immature megakaryocytes leading to platelet release and alleviation of thrombocytopenia. Thus, assays were designed to measure the emergence of these parameters in immature megakaryocyte cell lines, *i.e.*, CMK and DAMI cells. The CMK assay (Example 4) measures the appearance of a specific platelet marker, GPIIbIII<sub>a</sub>, and platelet shedding. The DAMI assay (Example 15) measures endoreplication since increases in ploidy are hallmarks of mature megakaryocytes. Recognizable megakaryocytes have ploidy values of 2N, 4N, 8N, 16N, 32N, etc. Finally, the *in vivo* mouse platelet rebound assay (Example 16) is useful in demonstrating that administration of the test compound (here the *mpl* ligand) results in elevation of platelet numbers.

Two additional *in vitro* assays have been developed to measure TPO activity. The first is a kinase receptor activation (KIRA) ELISA in which CHO cells are transfected with a *mpl*-Rse chimera and tyrosine phosphorylation of Rse is measured by ELISA after exposure of the *mpl* portion of the chimera to *mpl* ligand (see Example 17). The second is a receptor based ELISA in which ELISA plate coated rabbit anti-human IgG captures human chimeric receptor *mpl*-IgG which binds the *mpl* ligand being assayed. A biotinylated rabbit polyclonal antibody to *mpl* ligand (TPO155) is used to detect bound *mpl* ligand which is measured using streptavidin-peroxidase as described in Example 18.

#### 15. *In Vivo* Biological Response of Normal and Sublethally Irradiated Mice Treated with TPO

Both normal and sublethally irradiated mice were treated with truncated and full length TPO isolated from Chinese hamster ovary (CHO) cells, *E. coli*, and human embryonic kidney (293) cells. Both forms of TPO produced in these three hosts stimulated platelet production in mice, however, full length TPO isolated from CHO appeared to produce the greatest *in vivo* response. These results indicate that proper glycosylation of the carboxy-terminal domain may be necessary for optimal *in vivo* activity.

##### (a) *E. coli*-rhTPO(Met<sup>-1</sup>,153)

The "Met" form of the EPO domain (Met in the -1 position plus the first 153 residues of human TPO) produced in *E. coli* (see Example 23) was injected daily into

normal female C57 B6 mice as described in the legends to Figs. 25A, 25B and 25C. These figures show that the non-glycosylated truncated form of TPO produced in *E. coli* and refolded as described above is capable of stimulating about a two-fold increase in platelet production in normal mice without effecting the red or white blood cell population.

5 This same molecule injected daily into sublethally irradiated (<sup>137</sup>Cs) female C57 B6 mice as described in the legends to Figs. 26A, 26B and 26C stimulated platelet recovery and diminished nadir but had no effect on erythrocytes or leukocytes.

(b) CHO-rhTPO332

10 The full length form of TPO produced in CHO and injected daily into normal female C57 B6 mice as described in the legends to Figs. 27A, 27B and 27C produced about a five-fold increase in platelet production in normal mice without effecting the erythrocyte or leukocyte population.

15 (c) CHO-rhTPO332; *E. coli*-rhTPO(Met<sup>-1</sup>,153); 293-rhTPO332; and *E. coli*-rhTPO155

Dose response curves were constructed for treatment of normal mice with rhTPO from various cell lines (CHO-rhTPO332; *E. coli*-rhTPO(Met<sup>-1</sup>,153); 293-rhTPO332; and *E. coli*-rhTPO155) as described in the legend to Fig. 28. This figure shows that all tested forms of the molecule stimulate platelet production, however the 20 full length form produced in CHO has the greatest *in vivo* activity.

(d) CHO-rhTPO153, CHO-rhTPO"clipped" and CHO-rhTPO332

25 Dose response curves were also constructed for treatment of normal mice with various forms of rhTPO produced in CHO (CHO-rhTPO153, CHO-rhTPO"clipped" and CHO-rhTPO332) as described in the legend to Fig. 29. This figure shows that all tested CHO forms of the molecule stimulate platelet production, but that the full length 70 Kda form has the greatest *in vivo* activity.

**16. General Recombinant Preparation of *mpl* Ligand and Variants**

30 Preferably *mpl* ligand is prepared by standard recombinant procedures which involve production of the *mpl* ligand polypeptide by culturing cells transfected to express *mpl* ligand nucleic acid (typically by transforming the cells with an expression vector) and recovering the polypeptide from the cells. However, it is optionally envisioned that the *mpl* ligand may be produced by homologous 35 recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the *mpl* ligand. For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element may be inserted in the genome of the intended host cell in

5 proximity and orientation sufficient to influence the transcription of DNA encoding the desired *mpl* ligand polypeptide. The control element does not encode the *mpl* ligand, rather the DNA is indigenous to the host cell genome. One next screens for cells making the receptor polypeptide of this invention, or for increased or decreased levels of expression, as desired.

10 Thus, the invention contemplates a method for producing *mpl* ligand comprising inserting into the genome of a cell containing the *mpl* ligand nucleic acid molecule a transcription modulatory element in sufficient proximity and orientation to the nucleic acid molecule to influence transcription thereof, with an optional further step 15 comprising culturing the cell containing the transcription modulatory element and the nucleic acid molecule. The invention also contemplates a host cell containing the indigenous *mpl* ligand nucleic acid molecule operably linked to exogenous control sequences recognized by the host cell.

20 *A. Isolation of DNA Encoding *mpl* ligand Polypeptide*

25 The DNA encoding *mpl* ligand polypeptide may be obtained from any cDNA library prepared from tissue believed to possess the *mpl* ligand mRNA and to express it at a detectable level. The *mpl* ligand gene may also be obtained from a genomic DNA library or by *in vitro* oligonucleotide synthesis from the complete nucleotide or amino acid sequence.

30 Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind to the *mpl* ligand. For cDNA libraries suitable probes include oligonucleotides of about 20-80 bases in length that encode known or suspected portions of the *mpl* ligand cDNA from the same or different species; and/or complementary or homologous cDNAs or 35 fragments thereof that encode the same or a similar gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides, cDNAs, or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in Chapters 10-12 of Sambrook *et al.*, *supra*.

40 An alternative means to isolate the gene encoding *mpl* ligand is to use PCR methodology as described in section 14 of Sambrook *et al.*, *supra*. This method requires the use of oligonucleotide probes that will hybridize to DNA encoding the *mpl* ligand. Strategies for selection of oligonucleotides are described below.

45 A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably human or porcine kidney (adult or fetal) or liver cell lines. For example, human fetal

liver cell line cDNA libraries are screened with the oligonucleotide probes. Alternatively, human genomic libraries may be screened with the oligonucleotide probes.

5 The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually designed based on regions of the *mpl* ligand which have the least codon redundancy. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

10 The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ATP (e.g.,  $\gamma^{32}\text{P}$ ) and polynucleotide kinase to radiolabel the 5' end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

15 Of particular interest is the *mpl* ligand nucleic acid that encodes a full-length *mpl* ligand polypeptide. In some preferred embodiments, the nucleic acid sequence includes the native *mpl* ligand signal sequence. Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence.

20 *B. Amino Acid Sequence Variants of Native *mpl* ligand*

Amino acid sequence variants of *mpl* ligand are prepared by introducing appropriate nucleotide changes into the *mpl* ligand DNA, or by *in vitro* synthesis of the desired *mpl* ligand polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence for the porcine *mpl* ligand. For example, carboxy terminus portions of the mature full length *mpl* ligand may be removed by proteolytic cleavage, either *in vivo* or *in vitro*, or by cloning and expressing a fragment of the DNA encoding full length *mpl* ligand to produce a biologically active variant. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct 25 possesses the desired biological activity. The amino acid changes also may alter post-translational processes of the *mpl* ligand, such as changing the number or position of glycosylation sites. For the design of amino acid sequence variants of the *mpl* ligand, the location of the mutation site and the nature of the mutation will depend on the *mpl* ligand characteristic(s) to be modified. The sites for mutation can be modified 30 individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class 35 adjacent to the located site, or combinations of options 1-3.

A useful method for identification of certain residues or regions of the *mpl* ligand polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells, *Science*, 244:1081-1085 [1989]. Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by any, but preferably a neutral or negatively charged, amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed *mpl* ligand variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. For example, variants of the *mpl* ligand polypeptide include variants from the *mpl* ligand sequence, and may represent naturally occurring alleles (which will not require manipulation of the *mpl* ligand DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the *mpl* ligand characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Alternatively, amino acid sequence deletions for the *mpl* ligand may include a portion of or the entire carboxy-terminus glycoprotein domain. Amino acid sequence deletions may also include one or more of the first 6 amino-terminus residues of the mature protein. Optional amino acid sequence deletions comprise one or more residues in one or more of the loop regions that exist between the 'helical bundles'. Contiguous deletions ordinarily are made in even numbers of residues, but single or odd numbers of deletions are within the scope hereof. Deletions may be introduced into regions of low homology among the *mpl* ligands that share the most sequence identity to modify the activity of the *mpl* ligand. Or deletions may be introduced into regions of low homology among human *mpl* ligand and other mammalian *mpl* ligand polypeptides that share the most sequence identity to the human *mpl* ligand. Deletions from a mammalian *mpl* ligand polypeptide in areas of substantial homology with other mammalian *mpl* ligands will be more likely to modify the biological activity of the *mpl* ligand more

significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of *mpl* ligands in the affected domain, e.g., beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the mature *mpl* ligand sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. An exemplary preferred fusion is that of *mpl* ligand or fragment thereof and another cytokine or fragment thereof. Examples of terminal insertions include mature *mpl* ligand with an N-terminal methionyl residue, an artifact of the direct expression of mature *mpl* ligand in recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the mature *mpl* ligand molecule to facilitate the secretion of mature *mpl* ligand from recombinant hosts. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the *mpl* ligand molecule include the fusion to the N- or C-terminus of *mpl* ligand of immunogenic polypeptides (i.e., not endogenous to the host to which the fusion is administered), e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* *trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

A third group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the *mpl* ligand molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of *mpl* ligand and sites where the amino acids found in other analogues are substantially different in terms of side-chain bulk, charge, or hydrophobicity, but where there is also a high degree of sequence identity at the selected site among various *mpl* ligand species and/or within the various animal analogues of one *mpl* ligand member.

Other sites of interest are those in which particular residues of the *mpl* ligand obtained from various family members and/or animal species within one member are identical. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 3 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more

substantial changes, denominated exemplary substitutions in Table 3, or as further described below in reference to amino acid classes, are introduced and the products screened.

5

TABLE 3

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
10	Ala (A)	Val; Leu; Ile	Val
	Arg (R)	Lys; Gln; Asn	Lys
	Asn (N)	Gln; His; Lys; Arg	Gln
	Asp (D)	Glu	Glu
	Cys (C)	Ser	Ser
	Gln (Q)	Asn	Asn
15	Glu (E)	Asp	Asp
	Gly (G)	Pro	Pro
	His (H)	Asn; Gln; Lys; Arg	Arg
	Ile (I)	Leu; Val; Met; Ala; Phe; norleucine	Leu
	Leu (L)	norleucine; Ile; Val; Met; Ala; Phe	Ile
20	Lys (K)	Arg; Gln; Asn	Arg
	Met (M)	Leu; Phe; Ile	Leu
	Phe (F)	Leu; Val; Ile; Ala	Leu
	Pro (P)	Gly	Gly
	Ser (S)	Thr	Thr
25	Thr (T)	Ser	Ser
	Trp (W)	Tyr	Tyr
	Tyr (Y)	Trp; Phe; Thr; Ser	Phe
	Val (V)	Ile; Leu; Met; Phe; Ala; norleucine	Leu
30			

Substantial modifications in function or immunological identity of the *mpl* ligand are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the 35 substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr;
- (3) acidic: Asp, Glu;
- (4) basic: Asn, Gln, His, Lys, Arg;

5 (5) residues that influence chain orientation: Gly, Pro; and

- (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

In one embodiment of the invention, it is desirable to inactivate one or more protease cleavage sites that are present in the molecule. These sites are identified by inspection of the encoded amino acid sequence, in the case of trypsin, e.g., for an arginyl or lysinyl residue. When protease cleavage sites are identified, they are rendered inactive to proteolytic cleavage by substituting the targeted residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue.

In another embodiment, any methionyl residues other than the starting methionyl residue of the signal sequence, or any residue located within about three residues N- or C-terminal to each such methionyl residue, is substituted by another residue (preferably in accordance with Table 3) or deleted. Alternatively, about 1-3 residues are inserted adjacent to such sites.

Any cysteine residues not involved in maintaining the proper conformation of the *mpl* ligand also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. It has been found that the first and forth cysteines in the epo domain, numbered from the amino-terminus, are necessary for maintaining proper conformation but that the second and third are not. Accordingly, the second and third cysteines in the epo domain may be substituted.

Nucleic acid molecules encoding amino acid sequence variants of *mpl* ligand are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of *mpl* ligand polypeptide.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of *mpl* ligand DNA. This technique is well known in the art as described by Adelman *et al.*, *DNA*, 2:183 [1983]. Briefly, *mpl*

ligand DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of *mpl* ligand. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the *mpl* ligand DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea *et al.*, *Proc. Natl. Acad. Sci. USA*, 75:5765 [1978].

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera *et al.*, *Meth. Enzymol.*, 153:3 [1987]. Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, NY 1989).

Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the *mpl* ligand, and the other strand (the original template) encodes the native, unaltered sequence of the *mpl* ligand. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template, except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with *ExoIII* nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding *mp1* ligand mutants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid

substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of *mp*/ligand polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1  $\mu$ g) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50  $\mu$ l. The reaction mixture is overlayed with 35  $\mu$ l mineral oil. The reaction mixture is denatured for five minutes at 100°C, placed briefly on ice, and then 1  $\mu$ l *Thermus aquaticus* (*Taq*) DNA polymerase (5 units/ $\mu$ l, purchased from Perkin-Elmer Cetus) is added below the mineral oil layer.

The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows:

2 min. 55°C  
30 sec. 72°C, then 19 cycles of the following:  
5      30 sec. 94°C  
      30 sec. 55°C, and  
      30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform 10 (50:50 vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.*, *Gene*, 34:315 [1985]. The starting material is 15 the plasmid (or other vector) comprising the *mpl* ligand DNA to be mutated. The codon(s) in the *mpl* ligand DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate 20 locations in the *mpl* ligand DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard 25 techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated *mpl* ligand DNA sequence.

#### *C. Insertion of Nucleic Acid into a Replicable Vector*

30      The nucleic acid (e.g., cDNA or genomic DNA) encoding native or variant *mpl* ligand polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the nucleic acid to be inserted into 35 the vector, and (3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell with which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a

signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) *Signal Sequence Component*

The *mpl* ligand of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the *mpl* ligand DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native *mpl* ligand signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase, alpha factor, or acid phosphatase leaders, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (i.e., the *mpl* ligand presequence that normally directs secretion of *mpl* ligand from its native mammalian cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other *mpl* ligand polypeptides or from the same *mpl* ligand from a different animal species, signal sequences from a *mpl* ligand, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

(ii) *Origin of Replication Component*

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2  $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another

organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of *mpl* ligand DNA. However, the recovery of genomic DNA encoding *mpl* ligand is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the *mpl* ligand DNA.

*(iii) Selection Gene Component*

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, *J. Molec. Appl. Genet.*, 1:327 [1982]) mycophenolic acid (Mulligan *et al.*, *Science*, 209:1422 [1980]) or hygromycin Sugden *et al.*, *Mol. Cell. Biol.*, 5:410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Examples of other suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the *mpl* ligand nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes *mpl* ligand polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the

chromosomes of successive generations of recombinant cells. Increased quantities of *mpl* ligand are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 [1980]. The transformed cells are then exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding *mpl* ligand. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells [particularly wild-type hosts that contain endogenous DHFR] transformed or co-transformed with DNA sequences encoding *mpl* ligand, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 [1979]; Kingsman *et al.*, *Gene*, 7:141 [1979]; or Tschemper *et al.*, *Gene*, 10:157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 [1977]). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC No. 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

(iv) *Promoter Component*

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the *mpl* ligand nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the *mpl* ligand nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in

temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to *mpl* ligand encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native 5 *mpl* ligand promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the *mpl* ligand DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed *mpl* ligand as compared to the native *mpl* ligand promoter.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and 10 lactose promoter systems (Chang *et al.*, *Nature*, 275:615 [1978]; and Goeddel *et al.*, *Nature*, 281:544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 [1980] and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:21-15 25 [1983]). However, other known bacterial promoters are suitable. Their 15 nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding *mpl* ligand (Siebenlist *et al.*, *Cell*, 20:269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding *mpl* ligand polypeptide.

20 Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be 25 the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the 30 promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*, 255:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.*, 7:149 [1968]; and Holland, *Biochemistry*, 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

35 Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate

dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

5        *Mpl* ligand transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from 10 heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the *mpl* ligand sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an 15 SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, 273:113 [1978]; Mulligan and Berg, *Science*, 209:1422-1427 [1980]; Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:7398-7402 [1981]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hind*III E restriction fragment. Greenaway *et al.*, *Gene*, 18:355-360 [1982]. A 20 system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Gray *et al.*, *Nature*, 295:503-508 [1982] on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, 297:598-601 [1982] on expression of human  $\beta$ -interferon cDNA in mouse 25 cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, 79:5166-5170 [1982] on expression of the human interferon B1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:6777-6781 [1982] on expression of bacterial CAT 30 sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) *Enhancer Element Component*

Transcription of a DNA encoding the *mpl* ligand of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. 35 Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:993 [1981]) and 3' (Lusky *et al.*, *Mol. Cell Bio.*, 3:1108 [1983]) to the

transcription unit, within an intron (Banerji *et al.*, *Cell*, 33:729 [1983]), as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.*, 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer 5 from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297:17-18 [1982] on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a 10 position 5' or 3' to the *mpl* ligand encoding sequence, but is preferably located at a site 5' from the promoter.

15 (vi) *Transcription Termination Component*

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also 20 contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding *mpl* ligand.

25 (vii) *Construction and Analysis of Vectors*

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

30 For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC No. 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, *Nucleic Acids Res.*, 9:309 [1981] or by the method of Maxam *et al.*, *Methods in Enzymology*, 65:499 [1980].

35 (viii) *Transient Expression Vectors*

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the *mpl* ligand polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp.

16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are 5 particularly useful in the invention for purposes of identifying analogues and variants of *mpl* ligand polypeptide that have *mpl* ligand polypeptide biological activity.

(ix) *Suitable Exemplary Vertebrate Cell Vectors*

Other methods, vectors, and host cells suitable for adaptation to the synthesis of *mpl* ligand in recombinant vertebrate cell culture are described in Gething *et al.*, 10 *Nature*, 293:620-625 [1981]; Mantei *et al.*, *Nature*, 281:40-46 [1979]; Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of *mpl* ligand is pRK5 (EP 307,247 U. S. patent no. 5,258,287) or pSVI6B (PCT Publication No. WO 91/08291).

D. *Selection and Transformation of Host Cells*

15 Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryotic cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC No. 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC No. 31,537), and *E. coli* W3110 (ATCC No. 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

25 In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for *mpl* ligand encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290:140 [1981]; EP 139,383 published 2 May 1985), *Kluyveromyces* hosts (U.S. Patent No. 4,943,529) such as, e.g., *K. lactis* (Louvcourt *et al.*, *J. Bacteriol.*, 737 [1983]), *K. fragilis*, *K. bulgaricus*, *K. thermotolerans*, and *K. marxianus*, *yarrowia* [EP 402,226], *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, *J. Basic Microbiol.*, 28:265-278 [1988]), *Candida*, 30 *Trichoderma reesia* (EP 244,234), *Neurospora crassa* (Case *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:5259-5263 [1979]), and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, *Biochem. Biophys. Res.*

*Commun.*, 112:284-289 [1983]; Tilburn *et al.*, *Gene*, 26:205-221 [1983]; Yelton *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:1470-1474 [1984]) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4:475-479 [1985]).

Suitable host cells for the expression of glycosylated *mpl* ligand are derived 5 from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* 10 (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, e.g., Luckow *et al.*, *Bio/Technology*, 6:47-55 [1988]; Miller *et al.*, *Genetic Engineering*, Setlow *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, *Nature*, 315:592-594 [1985]. A variety of viral strains for transfection are publicly 15 available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation 20 with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the *mpl* ligand DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the *mpl* ligand is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the *mpl* ligand DNA. In addition, regulatory and signal sequences compatible 25 with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, *J. Mol. Appl. Gen.*, 1:561 [1982]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

30 However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors [1973]). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells 35 subcloned for growth in suspension culture, Graham *et al.*, *J. Gen. Virol.*, 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 [1980]); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 [1980]); monkey

kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, 383:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

5 Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or  
10 amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Successful transfection is generally recognized when any indication of  
15 the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as  
20 described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, *Gene*, 23:315 [1983] and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described  
25 in WO 91/00358 published 10 January 1991. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology*, 52:456-457 [1978] is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out  
30 according to the method of Van Solingen *et al.*, *J. Bact.*, 130:946 [1977] and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 76:3829 [1979]. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

#### *E. Culturing the Host Cells*

35 Prokaryotic cells used to produce the *mpl* ligand polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the *mpl* ligand of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10

(Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, 58:44 [1979], Barnes and Sato, *Anal. Biochem.*, 102:255 [1980], U.S. Patent No. 5 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985; or copending U.S.S.N. 07/592,107 or 07/592,141, both filed on 3 October 1990, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. 10 Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

15 The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

#### *F. Detecting Gene Amplification/Expression*

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77:5201-5205 25 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to 30 avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so 35 that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or

body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as 5 enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, *Am. J. Clin. Path.*, 75:734-738 [1980].

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal.

10 Conveniently, the antibodies may be prepared against a native *mpl* ligand polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further below.

*G. Purification of *mpl* ligand Polypeptide*

15 *Mpl* ligand preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When *mpl* ligand is expressed in a recombinant cell other than one of human origin, the *mpl* ligand is completely free of proteins or polypeptides of human origin. However, it is still usually necessary to purify *mpl* ligand from other recombinant 20 cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the *mpl* ligand *per se*. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. Alternatively, a commercially available protein concentration filter (e.g., Amicon or Millipore Pellicon ultrafiltration units) may be 25 used. The *mpl* ligand may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the *mpl* ligand is membrane bound. *Mpl* ligand thereafter is purified from contaminant soluble proteins and polypeptides by salting out and exchange or chromatographic procedures employing various gel matrices. These matrices include; acrylamide, agarose, dextran, cellulose 30 and others common to protein purification. Exemplary chromatography procedures suitable for protein purification include; immunoaffinity (e.g., anti-h*mpl* ligand Mab), receptoraffinity (e.g., *mpl*-IgG or protein A Sepharose), hydrophobic interaction chromatography (HIC) (e.g., ether, butyl, or phenyl Toyopearl), lectin chromatography (e.g., Con A-Sepharose, lentil-lectin-Sepharose), size exclusion 35 (e.g., Sephadex G-75), cation- and anion-exchange columns (e.g., DEAE or carboxymethyl- and sulfopropyl-cellulose), and reverse-phase high performance liquid chromatography (RP-HPLC) (see e.g., Urdal *et al.*, *J. Chromatog.*, 296:171 [1984] where two sequential RP-HPLC steps are used to purify recombinant human

IL-2). Other purification steps optionally include; ethanol precipitation; ammonium sulfate precipitation; chromatofocusing; preparative SDS-PAGE, and the like.

*Mpl* ligand variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native *mpl* ligand, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a *mpl* ligand fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal anti-*mpl* ligand column can be employed to absorb the *mpl* ligand variant by binding it to at least one remaining immune epitope. Alternatively, the *mpl* ligand may be purified by affinity chromatography using a purified *mpl*-IgG coupled to a (preferably) immobilized resin such as Affi-Gel 10 (Bio-Rad, Richmond, CA) or the like, by means well known in the art. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native *mpl* ligand may require modification to account for changes in the character of *mpl* ligand or its variants upon expression in recombinant cell culture.

20        *H. Covalent Modifications of mpl ligand Polypeptide*

Covalent modifications of *mpl* ligand polypeptides are included within the scope of this invention. Both native *mpl* ligand and amino acid sequence variants of the *mpl* ligand may be covalently modified. One type of covalent modification included within the scope of this invention is a *mpl* ligand fragment. Variant *mpl* ligand fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis or by enzymatic or chemical cleavage of the full-length or variant *mpl* ligand polypeptide. Other types of covalent modifications of the *mpl* ligand or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of the *mpl* ligand or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(5-imidazoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

5 Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-  
10 methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be  
15 performed in alkaline conditions because of the high  $pK_a$  of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic  
20 diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using  $^{125}I$  or  $^{131}I$  to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

25 Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ( $R-N=C=N-R'$ ), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholiny-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction  
30 with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking *mpI* ligand to a water-insoluble support matrix or surface for use in the method for purifying anti-*mpI* ligand antibodies, and *vice versa*. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide  
35 esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable

intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyan gen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for 5 protein immobilization.

Glutamyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

10 Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C- 15 terminal carboxyl group.

20 Another type of covalent modification of the *mpl* ligand polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native *mpl* ligand, and/or adding one or more glycosylation sites that are not 25 present in the native *mpl* ligand.

30 Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for 35 enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

30 Addition of glycosylation sites to the *mpl* ligand polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native *mpl* ligand sequence (for O-linked glycosylation 35 sites). For ease, the *mpl* ligand amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the *mpl* ligand polypeptide at preselected bases such that codons are generated that will translate into

the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of *mpl* Ligand."

Another means of increasing the number of carbohydrate moieties on the *mpl* ligand is by chemical or enzymatic coupling of glycosides to the polypeptide. These 5 procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulphydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or 10 (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 [1981].

Removal of carbohydrate moieties present on the *mpl* ligand polypeptide may be 15 accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al.*, *Arch. Biochem. Biophys.*, 259:52 [1987] and by Edge *et al.*, *Anal. Biochem.*, 118:131 [1981]. Enzymatic cleavage of carbohydrate moieties on polypeptides can 20 be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, *Meth. Enzymol.*, 138:350 [1987].

Glycosylation at potential glycosylation sites may be prevented by the use of the 25 compound tunicamycin as described by Duskin *et al.*, *J. Biol. Chem.*, 257:3105 [1982]. Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of *mpl* ligand comprises linking the *mpl* ligand polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. 30 Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. *Mpl* ligand polypeptides covalently linked to the forgoing polymers are referred to herein as pegylated *mpl* ligand polypeptides.

It will be appreciated that some screening of the recovered *mpl* ligand variant 35 will be needed to select the optimal variant for binding to a *mpl* and having the immunological and/or biological activity defined above. One can screen for stability in recombinant cell culture or in plasma (e.g., against proteolytic cleavage), high affinity to a *mpl* member, oxidative stability, ability to be secreted in elevated yields, and the like. For example, a change in the immunological character of the *mpl* ligand

polypeptide, such as affinity for a given antibody, is measured by a competitive-type immunoassay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, or susceptibility to proteolytic degradation are assayed by methods well known in the art.

5

### 17. General Methods for Preparation of Antibodies to *mpl* Ligand Antibody Preparation

#### (i) Polyclonal antibodies

10 Polyclonal antibodies to *mpl* ligand polypeptides or fragments are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the *mpl* ligand and an adjuvant. It may be useful to conjugate the *mpl* ligand or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, 15 for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N}=\text{C}=\text{NR}$ , where  $\text{R}$  and  $\text{R}^1$  are different alkyl groups.

20 Animals are immunized against the *mpl* ligand polypeptide or fragment, immunogenic conjugates or derivatives by combining 1 mg of 1  $\mu\text{g}$  of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 25 14 days later the animals are bled and the serum is assayed for *mpl* ligand antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal boosted with the conjugate of the same *mpl* ligand, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

30 (ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the 35 antibody as not being a mixture of discrete antibodies.

For example, the *mpl* ligand monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, *Nature*,

256:495 [1975], or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567 [Cabilly *et al.*]).

In the hybridoma method, a mouse or other appropriate host animal, such as hamster is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 [Academic Press, 1986]).

10 The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include 15 hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines 20 are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies 25 (Kozbor, *J. Immunol.*, 133:3001 [1984]; Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp.51-63, Marcel Dekker, Inc., New York, 1987).

Culture medium in which hybridoma cells are growing is assayed for 30 production of monoclonal antibodies directed against *mpl* ligand. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson & Pollard, *Anal. Biochem.*, 107:220 [1980].

35 After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-

1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

5 The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

10 DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA 15 also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, (Cabilly *et al.*, *supra*; Morrison, *et al.*, *Proc. Nat. Acad. Sci.*, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

20 Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a *mpl* ligand and another antigen-combining site having specificity for a 25 different antigen.

30 Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptopbutyrimidate.

35 For diagnostic applications, the antibodies of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g.,  $^{125}\text{I}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$ , or  $^3\text{H}$ , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter, *et al.*, *Nature*, 144:945 [1962]; David, *et al.*, *Biochemistry*, 13:1014 [1974]; Pain, *et al.*, *J. Immunol. Meth.*, 40:219 [1981]; and Nygren, *J. Histochem. and Cytochem.*, 5 30:407 [1982].

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

10 Competitive binding assays rely on the ability of a labeled standard (which may be a *mpl* ligand or an immunologically reactive portion thereof) to compete with the test sample analyte (*mpl* ligand) for binding with a limited amount of antibody. The amount of *mpl* ligand in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of 15 standard that becomes bound, the antibodies generally are immobilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein (*mpl* ligand) to be detected. 20 In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S. Patent No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is 25 labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme (e.g., horseradish peroxidase).

(iii) *Humanized and human antibodies*

Methods for humanizing non-human antibodies are well known in the art. 30 Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, 321:522-525 [1986]; Riechmann *et al.*, 35 *Nature*, 332:323-327 [1988]; Verhoeyen *et al.*, *Science*, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al.*, *supra*), wherein substantially less than an intact human

variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

5 The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the so called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as  
10 the human framework (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, 151:2296 [1993]; Chothia and Lesk, *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 [1992]; Presta *et al.*, *J. Immunol.*, 151:2623 [1993]).  
15

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using  
20 three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the  
25 residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and  
30 most substantially involved in influencing antigen binding. For further details see U.S. application Serial No. 07/934,373 filed 21 August 1992, which is a continuation-in-part of application Serial No. 07/715,272 filed 14 June 1991.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human  
35 antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J<sub>H</sub>) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line

immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551-255 [1993]; Jakobovits *et al.*, *Nature*, 362:255-258 [1993]; Brugermann *et al.*, *Year in Immuno.*, 7:33 [1993]. Human 5 antibodies can also be produced in phage display libraries (Hoogenboom and Winter, *J. Mol. Biol.* 227, 381 [1991]; Marks *et al.*, *J. Mol. Biol.* 222, 581 [1991]).

15 (iv) *Bispecific antibodies*

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. Methods 10 for making bispecific antibodies are known in the art.

Traditionally, the recombinant production of bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Millstein and Cuello, *Nature*, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light 15 chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in PCT publication No. WO 93/08829 (published 13 May 1993), and in 20 Traunecker *et al.*, *EMBO*, 10:3655-3659 [1991].

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, 25 CH<sub>2</sub> and CH<sub>3</sub> regions. It is preferred to have the first heavy chain constant region (CH<sub>1</sub>) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are cotransfected into a suitable host organism. This provides for great flexibility in 30 adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of 35 no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this

asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in copending application Serial No.

5 07/931,811 filed 17 August 1992.

For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 [1986].

(v) *Heteroconjugate antibodies*

Heteroconjugate antibodies are also within the scope of the present invention.

10 Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (PCT publication Nos. WO 91/00360 and WO 92/00373; EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 15 4,676,980, along with a number of cross-linking techniques.

**IV. Therapeutic Use of the Megakaryocytopoietic Protein *mpl* Ligand**

The biologically active *mpl* ligand having hematopoietic effector function and referred to here as a megakaryocytopoietic or thrombocytopoietic protein (TPO) may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia (e.g., aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the compounds of this invention as well as disorders such as disseminated intravascular coagulation (DIC), immune thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, and thrombotic thrombocytopenia. Additionally, 30 these megakaryocytopoietic proteins may be useful in treating myeloproliferative thrombocytotic diseases as well as thrombocytosis from inflammatory conditions and in iron deficiency.

Preferred uses of the megakaryocytopoietic or thrombocytopoietic protein (TPO) of this invention are in: myelotoxic chemotherapy for treatment of leukemia or solid tumors, myeloablative chemotherapy for autologous or allogeneic bone marrow transplant, myelodysplasia, Idiopathic aplastic anemia, congenital thrombocytopenia, and immune thrombocytopenia.

Still other disorders usefully treated with the megakaryocytopoietic proteins of this invention include defects or damage to platelets resulting from drugs, poisoning or activation on artificial surfaces. In these cases, the instant compounds may be employed to stimulate "shedding" of new "undamaged" platelets. For a more complete 5 list of useful applications, see the "Background" *supra*, especially section (a)-(f) and references cited therein.

The megakaryocytopoietic proteins of the instant invention may be employed alone or in combination with other cytokines, hematopoietins, interleukins, growth factors, or antibodies in the treatment of the above-identified disorders and conditions. 10 Thus, the instant compounds may be employed in combination with other protein or peptide having thrombopoietic activity including; G-CSF, GM-CSF, LIF, M-CSF, IL-1, IL-3, erythropoietin (EPO), kit ligand, IL-6, and IL-11.

The megakaryocytopoietic proteins of the instant invention are prepared in a mixture with a pharmaceutically acceptable carrier. This therapeutic composition can 15 be administered intravenously or through the nose or lung. The composition may also be administered parenterally or subcutaneously as desired. When administered systematically, the therapeutic composition should be pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art. Briefly, dosage formulations of 20 the compounds of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; 25 30 chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium and/or nonionic surfactants such as Tween, Pluronics or polyethyleneglycol.

About 0.5 to 500 mg of a compound or mixture of the megakaryocytopoietic protein as the free acid or base form or as a pharmaceutically acceptable salt, is 35 compounded with a physiologically acceptable vehicle, carrier, excipient, binder, preservative, stabilizer, flavor, etc., as called for by accepted pharmaceutical practice. The amount of active ingredient in these compositions is such that a suitable dosage in the range indicated is obtained.

Sterile compositions for injection can be formulated according to conventional pharmaceutical practice. For example, dissolution or suspension of the active compound in a vehicle such as water or naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Buffers, preservatives, antioxidants and the like can be incorporated according to accepted pharmaceutical practice.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.*, 15:167-277 [1981] and Langer, *Chem. Tech.*, 12:98-105 [1982] or poly(vinylalcohol)], polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers*, 22:547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through disulfide interchange, stabilization may be achieved by modifying sulphhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release megakaryocytopoietic protein compositions also include liposomally entrapped megakaryocytopoietic protein. Liposomes containing megakaryocytopoietic protein are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:3688-3692 [1985]; Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4030-4034 [1980]; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is

greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal megakaryocytopoietic protein therapy.

The dosage will be determined by the attending physician taking into consideration various factors known to modify the action of drugs including severity 5 and type of disease, body weight, sex, diet, time and route of administration, other medications and other relevant clinical factors. Typically, the daily regimen will range from 0.1-100  $\mu$ g/kg body weight. Preferably the dosage will range from 0.1-50  $\mu$ g/kg body weight. More preferably, the initial dosage will range from 1 to 5  $\mu$ g/kg/day. Optionally, the dosage range will be the same as that of other cytokines, 10 especially G-CSF, GM-CSF, and EPO. Therapeutically effective dosages may be determined by either *in vitro* or *in vivo* methods.

#### EXAMPLES

Without further description, it is believed that one of ordinary skill in the art 15 can, using the preceding description and illustrative examples, make and utilize the present invention to the fullest extent. The following working examples therefore specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way of the remainder of the disclosure.

#### 20 EXAMPLE 1

##### *Partial Purification of the Porcine mpl Ligand*

Platelet-poor plasma was collected from normal or aplastic anemic pigs. Pigs were rendered aplastic by irradiation with 900 cGy of total body irradiation using a 4mEV linear accelerator. The irradiated pigs were supported for 6-8 days with 25 intramuscular injections of cefazolin. Subsequently, their total blood volume was removed under general anesthesia, heparinized, and centrifuged at 1800  $\times$  g for 30min. to make platelet-poor plasma. The megakaryocyte stimulating activity was found to peak 6 days after irradiation.

Aplastic porcine plasma obtained from irradiated pigs is made 4M with NaCl 30 and stirred for 30 min. at room temperature. The resultant precipitate is removed by centrifugation at 3800 rpm in a Sorvall RC3B and the supernatant is loaded onto a Phenyl-Toyopearl column (220 ml) equilibrated in 10 mM NaPO<sub>4</sub> containing 4M NaCl. The column is washed with this buffer until A<sub>280</sub> is <0.05 and eluted with dH<sub>2</sub>O. The eluted protein peak is diluted with dH<sub>2</sub>O to a conductivity of 15mS and 35 loaded onto a Blue-Sepharose column equilibrated (240 ml) in PBS. Subsequently, the column is washed with 5 column volumes each of PBS and 10mM NaPO<sub>4</sub> (pH 7.4) containing 2M urea. Proteins are eluted from the column with 10mM NaPO<sub>4</sub> (pH 7.4) containing 2M urea and 1M NaCl. The eluted protein peak is made 0.01% octyl

glucosid (n-octyl  $\beta$ -D-glucopyranoside) and 1 mM aCh with EDTA and Pefabloc (Boehringer Mannheim) and loaded directly onto tandemly linked CD4-IgG (Capon, D.J. et al. *Nature* 337:525-531 [1989]) and *mpl*-IgG Ultralink (Pierce) columns (see below). The CD4-IgG (2 ml) column is removed after the sample is loaded and the 5 *mpl*-IgG (4 ml) column is washed with 10 column volumes each of PBS and PBS containing 2 M NaCl and eluted with 0.1M glycine-HCl pH 2.25. Fractions are collected into 1/10th volume 1M Tris-HCl (pH 8.0).

Analysis of eluted fractions from the *mpl*-affinity column by SDS-PAGE (4-20%, Novex gel) run under reducing conditions, revealed the presence of several 10 proteins (Fig. 5). Proteins that silver stain with the strongest intensity resolve with apparent Mr of 66,000, 55,000, 30,000, 28,000 and 14,000. To determine which of these proteins stimulate proliferation of Ba/F3-*mpl* cell cultures these proteins were eluted from the gel as described in Example 2 below.

#### *Ultralink Affinity Columns*

15 10-20 mg of *mpl*-IgG or CD4-IgG in PBS are coupled to 0.5 grams of Ultralink resin (Pierce) as described by the manufacturer's instructions.

#### *Construction and Expression of *mpl*-IgG*

A chimeric molecule comprising the entire extracellular domain of human *mpl* (amino acids 1-491) and the Fc region of a human IgG1 molecule was expressed in 20 293 cells. A cDNA fragment encoding amino acids 1-491 of human *mpl* was obtained by PCR from a human megakaryocytic CMK cell cDNA library and sequenced. A Clal site was inserted at the 5' end and a BstEII site at the 3' end. This fragment was cloned upstream of the IgG1 Fc coding region in a Bluescript vector between the Clal and the BstEII sites after partial digestion of the PCR product with BstEII because of two other 25 BstEII sites present in the DNA encoding the extracellular domain of *mpl*. The BstEII site introduced at the 3' end of the *mpl* PCR product was designed to have the Fc region in frame with the *mpl* extracellular domain. The construct was subcloned into pRK5-tkneo vector between the Clal and XbaI sites and transfected into 293 human embryonic kidney cells by the calcium phosphate method. The cells were selected in 0.4 mg/ml 30 G418 and individual clones were isolated. *Mpl*-IgG expression from isolated clones was determined using a human Fc specific ELISA. The best expression clone had an expression level of 1-2 mg/ml of *mpl*-IgG.

#### *Ba/F3 *mpl* P Expressing Cells*

A cDNA corresponding to the entire coding region of human *mpl* P was cloned 35 into pRK5-tkneo which was subsequently linearized with NotI and transfected into the IL-3 dependent cell line Ba/F3 by electroporation ( $1 \times 10^7$  cells, 9605F, 250Volts). Three days later selection was started in the presence of 2 mg/ml of G418. The cells were selected as pools or individual clones were obtained by limiting dilution in 96

well plates. Selected cells were maintained in RPMI containing 15% FBS, 1mg /ml G418, 20mM Glutamine, 10mM HEPES and 100  $\mu$ g/ml of Pen-Strep. Expression of *mpl* P in selected clones was determined by FACS analysis using a anti-*mpl* P rabbit polyclonal antibody.

5 *Ba/F3 mpl ligand Assay*

The *mpl* ligand assay was conducted as shown in Fig. 2. To determine the presence of *mpl* ligand from various sources, the *mpl* P Ba/F3 cells were starved of IL-3 for 24 hours at a cell density of  $5 \times 10^5$  cells/ml in a humidified incubator at 37°C in 5% CO<sub>2</sub> and air. Following IL-3 starvation the cells were plated out in 96 10 well culture dishes at a density of 50,000 cells in 200  $\mu$ l of media with or without diluted samples and cultured for 24 hrs in a cell culture incubator. 20  $\mu$ l of serum free RPMI media containing 1  $\mu$ Cl of <sup>3</sup>H-thymidine was added to each well for the last 6-8 hours. The cells were then harvested on 96 well GF/C filter plates and washed 5 times with water. The filters were counted in the presence of 40  $\mu$ l of scintillation 15 fluid (Microscint 20) in a Packard Top Count counter.

**EXAMPLE 2**

*Highly Purified Porcine mpl Ligand*  
*Gel Elution Protocol*

20 Equal amounts of affinity purified *mpl* ligand (fraction 6 eluted from the *mpl*-IgG column) and 2X Laemmli sample buffer were mixed at room temperature without reducing agent and loaded onto a Novex 4-20% polyacrylamide gel as quickly as possible. The sample was not heated. As a control, sample buffer without ligand was run in an adjacent lane. The gel was run at 4-6°C at 135 volts for approximately 2 25 1/4 hours. The running buffer was initially at room temperature. The gel was then removed from the gel box and the plate on one side of the gel removed.

A replica of the gel was made on nitrocellulose as follows: A piece of nitrocellulose was wet with distilled water and carefully laid on top of the exposed gel face so air bubbles were excluded. Fiducial marks were placed on the nitrocellulose 30 and the gel plate so the replica could be accurately repositioned after staining. After approximately 2 minutes, the nitrocellulose was carefully removed, and the gel was wrapped in plastic wrap and placed in the refrigerator. The nitrocellulose was stained with Biorad's gold total protein stain by first agitating it in 3 x 10 ml 0.1% Tween 20 + 0.5 M NaCl + 0.1 M Tris-HCl pH 7.5 over approximately 45 minutes followed by 3 35 x 10 ml purified water over 5 minutes. The gold stain was then added and allowed to develop until the bands in the standards were visible. The replica was then rinsed with water, placed over the plastic wrap on the gel and carefully aligned with the fiducial marks. The positions of the Novex standards were marked on the gel plate and lines

were drawn to indicate the cutting positions. The nitrocellulose and plastic wrap were then moved and the gel cut along the indicated lines with a sharp razor blade. The cuts were extended beyond the sample lanes so they could be used to determine the positions of the slices when the gel was stained. After the slices were removed, the 5 remaining gel was silver stained and the positions of the standards and the cut marks were measured. The molecular weights corresponding to the cut positions were determined from the Novex standards.

The 12 gel slices were placed into the cells in two Biorad model 422 electroeluters. 12-14K molecular weight cutoff membrane caps were used in the cells. 10 50 mM ammonium bicarbonate + 0.05% SDS (approximately pH 7.8) was the elution buffer. One liter of buffer was chilled approximately 1 hour in a 4-6°C coldroom before use. Gel slices were eluted at 10 mA/cell (40 v initially) in a 4-6°C coldroom. Elution took approximately 4 hours. The cells were then carefully removed and the liquid above the frit removed with a pipet. The elution chamber was removed and any 15 liquid above the membrane cap removed with a pipet. The liquid in the membrane cap was removed with a Pipetman and saved. Fifty  $\mu$ l aliquots of purified water were then placed in the cap, agitated and removed until all the SDS crystals dissolved. These washes were combined with the saved liquid above. Total elution sample volume was 300-500  $\mu$ l per gel slice. Samples were placed in 10 mm Spectrapor 4 12-14K 20 cutoff dialysis tubing which had been soaked several hours in purified water. They were dialyzed overnight at 4-6°C against 600 ml of phosphate buffered saline (PBS is approximately 4 mM in potassium) per 6 samples. The buffer was replaced the next morning and dialysis continued for 2.5 hours. Samples were then removed from the dialysis bags and placed in microfuge tubes. The tubes were placed on ice for 1 hour, 25 microfuged at 14K rpm for 3 min. and the supernatants carefully removed from the precipitated SDS. The supernatants were then placed on ice for approximately 1 hour more and microfuged again for 4 min. The supernatants were diluted in phosphate buffered saline and submitted for the activity assay. Remaining samples were frozen at -70°C.

30

### EXAMPLE 3

#### *Porcine mpl Ligand Microsequencing*

Fraction 6 (2.6 ml) from the *mpl*-IgG affinity column was concentrated on a 35 Microcon-10 (Amicon). In order to prevent the *mpl* ligand from absorbing to the Microcon, the membrane was rinsed with 1% SDS and 5  $\mu$ l of 10 % SDS was added to fraction 6. Sample buffer (20  $\mu$ l) of 2X was added to the fraction #6 after Microcon concentration (20  $\mu$ l) and the total volume (40  $\mu$ l) was loaded on a single lane of a 4-20 % gradient acrylamide gel (Novex). The gel was run following Novex protocol. The

gel was then equilibrated for 5 min. prior to electroblotting in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0, containing 10% methanol. Electroblotting onto Immobilon-PSQ membranes (Millipore) was carried out for 45 min. at 250 mA constant current in a BioRad Trans-Blot transfer cell (32). The PVDF membrane was stained with 0.1% Coomassie Blue R-250 in 40% methanol, 0.1% acetic acid for 1 min. and destained for 2-3 min. with 10% acetic acid in 50% methanol. The only proteins that were visible in the Mr 18,000-35,000 region of the blot had Mr of 30,000, 28,000 and 22,000.

Bands at 30, 28 and 22 kDa were subjected to protein sequencing. Automated protein sequencing was performed on a model 470A Applied Biosystem sequencer equipped with an on-line PTH analyzer. The sequencer was modified to inject 80-90% of the sample (Rodriguez, *J. Chromatogr.*, 350:217-225 [1985]). Acetone (~12  $\mu$ l/l) was added to solvent A to balance the UV absorbance. Electroblotted proteins were sequenced in the Blott cartridge. Peaks were integrated with Justice Innovation software using Nelson Analytical 970 Interfaces. Sequence interpretation was performed on a VAX 5900 (Henzel *et al.*, *J. Chromatogr.*, 404:41-52 [1987]). N-terminal sequences (using one letter code with uncertain residues in parenthesis) and quantity of material obtained (in brackets) is presented in Table 2'.

20

TABLE 2'  
*Mpl Ligand Amino-Terminus Sequences*

30 kDa [1.8 pmol]	1 5 10 15 20 25	(S) P A P P A(C)D P R L L N K L L R D D (H/S) V L H (G) R L	(SEQ ID NO: 30)
28 kDa [0.5 pmol]	1 5 10 15 20 25	(S) P A P P A X D P R L L N K L L R D D (H) V L (H) G R	(SEQ ID NO: 31)
18-22 kDa [0.5 pmol]	1 5 10	X P A P P A X D P R L X (N) (K)	(SEQ ID NO: 32)

#### EXAMPLE 4

##### *Liquid Suspension Megakaryocytopoiesis Assay*

25 Human peripheral stem cells (PSC) (obtained from consenting patients) were diluted 5 fold with IMDM media (Gibco) and centrifuged for 15 min. at room temp. at 800 x g. The cell pellets were resuspended in IMDM and layered onto 60% Percoll (density 1.077 gm/ml) (Pharmacia) and centrifuged at 800 x g for 30 min. The

light density mononuclear cells were aspirated at the interface and washed 2x with IMDM and plated out at 1-2 x 10<sup>6</sup> cells/ml in IMDM containing 30% FBS (1 ml final volume) in 24 well tissue culture clusters (Costar). APP or *mpl* ligand depleted APP was added to 10% and cultures were grown for 12-14 days in a humidified incubator 5 at 37°C in 5% CO<sub>2</sub> and air. The cultures were also grown in the presence of 10% APP with 0.5 µg of *mpl*-IgG added at days 0, 2 and 4. APP was depleted of *mpl* ligand by passing APP through a *mpl*-IgG affinity column.

To quantitate megakaryocytopoiesis in these liquid suspension cultures, a modification of Solberg *et al.* was used and employs a radiolabeled murine IgG 10 monoclonal antibody (HP1-1D) to GPIIb/IIIa (provided by Dr. Nichols, Mayo Clinic). 100 µg of HP1-1D (see Grant, B. *et al.*, *Blood* 69:1334-1339 [1987]). was radiolabeled with 1mCi of Na<sup>125</sup>I using Enzymobeads (Biorad, Richmond, CA) as described by the manufacturer's instructions. Radiolabeled HP1-1D was stored at -70°C in PBS containing 0.01% octyl-glucoside. Typical specific activities were 1-2 15 x 10<sup>6</sup> cpm/µg (>95% precipitated by 12.5% trichloroacetic acid ).

Liquid suspension cultures were set up in triplicate for each experimental point. After 12-14 days in culture the 1ml cultures were transferred to 1.5ml eppendorf tubes and centrifuged at 800 x g for 10 min. at room temp. and the resultant cell pellets were resuspended in 100 µl of PBS containing 0.02% EDTA and 20% 20 bovine calf serum. 10ng of <sup>125</sup>I-HP1-1D in 50 µl of assay buffer was added to the resuspended cultures and incubated for 60 min. at room temperature (RT) with occasional shaking. Subsequently, cells were collected by centrifugation at 800 x g for 10 min. at RT and washed 2x with assay buffer. The pellets were counted for 1 min. in a gamma counter (Packard). Non-specific binding was determined by adding 1 µg of 25 unlabeled HP1-1D for 60 min. before the addition of labeled HP1-1D. Specific binding was determined as the total <sup>125</sup>I-HP1-1D bound minus that bound in the presence of excess unlabeled HP1-1D.

#### EXAMPLE 5

##### 30 *Oligonucleotide PCR Primers*

Based on the amino-terminal amino acid sequence obtained from the 30 kDa, 28 kDa and 18-22 kDa proteins, degenerate oligonucleotides were designed for use as polymerase chain reaction (PCR) primers (see Table 4). Two primer pools were synthesized, a positive sense 20 mer pool encoding amino acid residues 2-8 (*mpl* 1) 35 and an anti-sense 21-mer pool complimentary to sequences encoding amino acids 18-24 (*mpl* 2).

TABLE 4

## Degenerate Oligonucleotide Primer Pools

<i>mpl</i> 1:5' CCN GCN CCN CCN GCN TGY GA 3' (2,048-fold degenerate)	(SEQ ID NO: 35)
<i>mpl</i> 2:5' NCC RTG NAR NAC RTG RTC RTC 3' (2,048-fold degenerate)	(SEQ ID NO: 36)

Porcine genomic DNA, isolated from porcine peripheral blood lymphocytes, was  
 5 used as a template for PCR. The 50  $\mu$ l reaction contained: 0.8  $\mu$ g of porcine genomic  
 DNA in 10mM Tris-HCl (pH 8.3), 50mM KCl, 3mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA, 400  $\mu$ M  
 dNTPs, 1  $\mu$ M of each primer pool and 2.5 units of *Taq* polymerase. Initial template  
 10 denaturation was at 94°C for 8 min. followed by 35 cycles of 45 seconds at 94°C, 1  
 min. at 55°C and 1 min. at 72°C. The final cycle was allowed to extend for 10 min. at  
 15 72°C. PCR products were separated by electrophoresis on a 12% polyacrylamide gel  
 and visualized by staining with ethidium bromide. It was reasoned that if the amino-  
 terminal amino acid sequence was encoded by a single exon then the correct PCR  
 product was expected to be 69 bp. A DNA fragment of this size was eluted from the gel  
 and subcloned into pGEMT (Promega). Sequences of three clones are shown below in  
 Table 5.

TABLE 5

## 69 bp Porcine Genomic DNA Fragments

<b>gemT3</b>	
5'CCAGCGCCGC CAGCCTGTGA CCCCGACTC CTAATAAAC TGCTCGTGA	
3'GGTCGCGGCG GTCGGACACT GGGGGCTGAG GATTATTTG ACGGAGCACT	
TGACCACGTT CAGCACGGC [69 bp] (SEQ ID NO: 37)	
ACTGGTGCAA GTCGTGCCG (SEQ ID NO: 38)	
<b>gemT7</b>	
5'CCAGCACCTC CGGCATGTGA CCCCGACTC CTAATAAAC TGCTCGTGA	
3'GGTCGTGGAG GCCGTACACT GGGGGCTGAG GATTATTTG ACGAAGCACT	
CGACCACGTC CATCACGGC [69 bp] (SEQ ID NO: 39)	
GCTGGTGCAAG GTAGTGCCG (SEQ ID NO: 40)	

gemT9

P R L L N K L L R (SEQ ID

NO: 32)

5' CCAGCACCGCCGGCATGTGACCCCCGACTCCTAAATAACTGCTTCGTGACG  
3' GGTCGTGGCGGCCGTACACTGGGGCTGAGGATTATTTGACGAAGCACTGC

ATCATGTCTATCACGGT 3' (SEQ ID NO: 41)

TAGTACAGATAGTGCCA 5' (SEQ ID NO: 42)

The position of the PCR primers is indicated by the underlined bases. These results verify the N-terminal sequence obtained for amino acids 9-17 for the 30 kDa, 28 kDa and 18-22 kDa proteins and indicated that this sequence is encoded by a single exon of

5 porcine DNA.

## EXAMPLE 6

*Human mpl Ligand Gene*

Based on the results from Example 5, a 45-mer deoxyoligonucleotide, called  
10 pR45, was designed and synthesized to screen a genomic library. The 45-mer had the  
following sequence:

5' GCC-GTG-AAG-GAC-GTG-GTC-GTC-ACG-AAG-CAG-TTT-ATT-TAG-GAG-TCG 3'  
(SEQ ID NO: 28)

This oligonucleotide was  $^{32}\text{P}$ -labeled with ( $\gamma$  $^{32}\text{P}$ )-ATP and T4 kinase and used  
15 to screen a human genomic DNA library in  $\lambda$ gem12 under low stringency hybridization  
and wash conditions (see Example 7). Positive clones were picked, plaque purified  
and analyzed by restriction mapping and southern blotting. Clone #4 was selected for  
additional analysis.

A 2.8 kb BamHI-XbaI fragment that hybridized to the 45-mer was subcloned  
20 into pBluescript SK-. Partial DNA sequencing of this clone was performed using as  
primers oligonucleotides specific to the porcine *mpl* ligand DNA sequence. The  
sequence obtained confirmed that DNA encoding the human homolog of the porcine *mpl*  
ligand had been isolated. An EcoRI restriction site was detected in the sequence  
allowing us to isolate a 390 bp EcoRI-XbaI fragment from the 2.8 kb BamHI-XbaI and  
25 to subclone it in pBluescript SK-.

Both strands of this fragment were sequenced. The human DNA sequence and  
deduced amino acid sequence are shown in Fig. 9 (SEQ ID NOS: 3 & 4). The predicted  
positions of introns in the genomic sequence are also indicated by arrows, and define a  
putative exon ("exon 3").

Examination of the predicted amino acid sequence confirms that a serine residue is the first amino acid of the matur *mpl* ligand, as determined from direct amino acid sequence analysis. Immediately upstream from this codon the predicted amino acid sequence is highly suggestive of a signal sequence involved in secretion of the mature *mpl* ligand. This signal sequence coding region is probably interrupted at nucleotide position 68 by an intron.

In the 3' direction the exon appears to terminate at nucleotide 196. This exon therefore encodes a sequence of 42 amino acids, 16 of which are likely to be part of a signal sequence and 26 of which are part of the mature human *mpl* ligand.

10

#### EXAMPLE 7

##### *Full Length Human mpl Ligand cDNA*

Based on the human "exon 3" sequence (Example 6) two non-degenerate oligonucleotides corresponding to the 3' and 5' ends of the "exon 3" sequence were 15 synthesized (Table 6).

TABLE 6

##### Human cDNA Non-degenerate PCR Oligonucleotide Primers

Fwd primer: 5' GCT AGC TCT AGA AAT TGC TCC TCG TGG TCA TGC TTC T 3'	(SEQ ID NO: 43 )
Rvs primer: 5' CAG TCT GCC GTG AAG GAC ATG G 3'	(SEQ ID NO: 44 )

These two primers were used in PCR reactions employing as a template DNA from various human cDNA libraries or 1 ng of Quick Clone cDNA (Clonetech) from 20 various tissues using the conditions described in the Example 5. The expected size of the correct PCR product was 140 bp. After analysis of the PCR products on a 12% polyacrylamide gel, a DNA fragment of the expected size was detected in cDNA libraries prepared from adult kidney, 293 fetal kidney cells and cDNA prepared from human fetal liver (Clonetech cat. #7171-1).

25 A fetal liver cDNA library in  $\lambda$  DR2 (Clonetech cat. # HL1151x) was screened with the same 45 mer oligonucleotide used to screen the human genomic library. The oligonucleotide was labelled with ( $\gamma$ <sup>32</sup>P)-ATP using T4 polynucleotide kinase. The library was screened under low stringency hybridization conditions. The filters were prehybridized for 2hr then hybridized with the probe overnight at 42°C in 20% formamide, 5xSSC, 10xDenhardt's, 0.05M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50  $\mu$ g/ml of sonicated salmon sperm DNA for 16hr. Filters were then rinsed in 2xSSC and then washed once in 0.5xSSC, 0.1% SDS at 42°C. Filters were exposed overnight to Kodak X-Ray film. Positive clones were picked, plaque purified

and the insert size was determined by PCR using oligonucleotides flanking the BamHI-XbaI cloning in  $\lambda$  DR2 (Clonetech cat. #6475-1). 5  $\mu$ l of phage stock was used as a template source. Initial denaturation was for 7 min. at 94°C followed by 30 cycles of amplification (1 min. at 94°C, 1 min. at 52°C and 1.5 min. at 72°C). Final extention 5 was for 15 min. at 72°C. Clone # FL2b had a 1.8kb insert and was selected for further analysis.

The plasmid pDR2 (Clonetech,  $\lambda$ DR2 & pDR2 cloning and Expression System Library Protocol Handbook, p 42) contained within the  $\lambda$ DR2 phage arms, was rescued as described per manufacturer's instructions (Clonetech,  $\lambda$ DR2 & pDR2 cloning and 10 Expression System Library Protocol Handbook, p 29-30). Restriction analysis of the plasmid pDR2-FL2b with BamHI and XbaI indicated the presence of an internal BamHI restriction site in the insert approximately at position 650. Digestion of the plasmid with BamHI-XbaI cut the insert in two fragments, one of 0.65 kb and one of 1.15 kb. DNA sequence was determined with three different classes of template derived from the 15 plasmid pDR2-FL2b. DNA sequencing of double-stranded plasmid DNA was carried out with the ABI373 (Applied Biosystems, Foster City, California) automated fluorescent DNA sequencer using standard protocols for dye-labeled dideoxy nucleoside triphosphate terminators (dye-terminators) and custom synthesized walking primers (Sanger *et al.*, *Proc. Natl. Acad. Sci. USA*, 74:5463-5467 [1977]; Smith *et al.*, 20 *Nature*, 321:674-679 [1986]). Direct sequencing of polymerase chain reaction amplified fragments from the plasmid was done with the ABI373 sequencer using custom primers and dye-terminator reactions. Single stranded template was generated with the M13 Janus vector (DNASTAR, Inc., Madison, Wisconsin) (Burland *et al.*, *Nucl. Acids Res.*, 21:3385-3390 [1993]). BamHI-XbaI (1.15 kb) and BamHI (0.65 25 kb) fragments were isolated from the plasmid pDR2-FL2b, the ends filled in with T4 DNA polymerase in the presence of deoxynucleotides, and then subcloned into the SmaI site of M13 Janus. Sequencing was carried out with standard protocols for dye-labeled M13 universal and reverse primers, or walking primers and dye-terminators. Manual sequencing reactions were carried out on single strand M13 DNA using walking 30 primers and standard dideoxy-terminator chemistry (Sanger *et al.*, *Proc. Natl. Acad. Sci. USA*, 74:5463-5467 [1977]),  $^{33}P$ -labeled  $\alpha$ -dATP and Sequenase (United States Biochemical Corp., Cleveland, Ohio). DNA sequence assembly was carried out with Sequencher V2.1b12 (Gene Codes Corporation, Ann Arbor, Michigan). The nucleotide and deduced sequences of hML are provided in Fig. 1 (SEQ ID NO: 1).

**EXAMPLE 8*****Isolation of the Human *mpl* Ligand (TPO) Gene.***

Human genomic DNA clones of the TPO gene were isolated by screening a human genomic library in  $\lambda$ -Gem12 with pR45, a previously described oligonucleotide probe under low stringency conditions (see Example 7) or under high stringency conditions with a fragment corresponding to the 3' half of human cDNA coding for the *mpl* ligand (from the BamH1 site to the 3' end). Two overlapping lambda clones spanning 35 kb were isolated. Two overlapping fragments (BamH1 and EcoRI) containing the entire TPO gene were subcloned and sequenced. The structure of the human gene is composed of 6 exons within 7 kb of genomic DNA (Fig. 14 A, B and C). The boundaries of all exon/intron junctions are consistent with the consensus motif established for mammalian genes (Shapiro, M. B., et al., *Nucl. Acids Res.* 15:7155 [1987]). Exon 1 and exon 2 contain 5' untranslated sequence and the initial four amino acids of the signal peptide. The remainder of the secretory signal and the first 26 amino acids of the mature protein are encoded within exon 3. The entire carboxyl domain and 3' untranslated as well as ~50 amino acids of the erythropoietin-like domain are encoded within exon 6. The four amino acids involved in the deletion observed within hML-2 (hTPO-2) are encoded at the 5' end of exon 6.

20

**EXAMPLE 9*****Transient Expression of Human *mpl* Ligand (hML)***

In order to subclone the full length insert contained in pDR2-FL2b, the plasmid was digested with XbaI to completion, then partially digested with BamHI. A DNA fragment corresponding to the 1.8 kb insert was gel purified and subcloned in pRK5 (pRK5-hmp/ I) (see U.S. Patent No. 5,258,287 for construction of pRK5) under the control of the cytomegalovirus immediate early promoter. DNA from the construct pRK5-hmp/ I was prepared by the PEG method and transfected in Human embryonic kidney 293 cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with F-12 nutrient mixture, 20 mM Hepes (pH 7.4) and 10% fetal bovine serum. Cells were transfected by the calcium phosphate method as described (Gorman, C. [1985] in *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. II, pp. 143-190, IRL Press, Washington, D. C.). 36 h after transfection, the supernatant of the transfected cells was assayed for activity in the proliferation assay (see Example 1). Supernatant of 293 cells transfected with pRK vector only gave no stimulation of the Ba/F3 or Ba/F3-*mpl* cells (Fig. 12A). Supernatant of cells transfected with pRK5-hmp/ I had no effect on the Ba/F3 cells but dramatically stimulates the proliferation of Ba/F3-*mpl* cells (Fig. 12A), indicating that this cDNA encodes a functionally active human *mpl* ligand.

## EXAMPLE 10

*Human Mpl Ligand Isoforms**hML2, hML3, and hML4*

5 In order to identify alternatively spliced forms of hML, primers were synthesized corresponding to each end of the coding sequence of hML. These primers were employed in RT-PCR to amplify human adult liver RNA. Additionally, internal primers flanking selected regions of interest (see below) were constructed and similarly employed. Direct sequencing of the ends of the PCR product revealed a single  
 10 sequence corresponding exactly to the sequence of the cDNA isolated from the human fetal liver library (see Fig. 1 [SEQ ID NO: 1]). However, a region near the C-terminus of the EPO-domain (in the middle of the PCR product) exhibited a complex sequence pattern suggesting the existence of possible splice variants in that region. To isolate these splice variants, the primers provided in Table 7 flanking the region of  
 15 interest were used in a PCR as templates for human adult liver cDNA.

TABLE 7

## Human ML Isoform PCR Primers

phmplcdna.3e1:	5'TGTGGACTTAGCTGGAGAATG3'	(SEQ ID NO: 45)
pbx4.f2:	5'GGTCCAGGGACCTGGAGGTTG3'	(SEQ ID NO: 46)

20 The PCR products were subcloned blunt into M13. Sequencing of individual subclones revealed the existence of at least 3 ML isoforms. One of them, hML (also referred to as hML332), is the longest form and corresponds exactly to the sequence isolated from the fetal liver library. Sequences of the four human *mpl* ligand isoforms listed from longest (hML) to shortest (hML-4) are provided in (Fig. 11 [SEQ ID NOS: 6, 8, 9 & 10]).

25

## EXAMPLE 11

*Construction and Transient Expression of Human Mpl Ligand Isoforms and Substitutional Variants**hML2, hML3, and hML(R153A, R154A)*

30 Isoforms hML2 and hML3 and substitutional variant hML(R153A, R154A) were reconstituted from hML using the recombinant PCR technique described by Russell Higuchi, in PCR Protocols, *A guide to Methods and Applications*, Acad. Press, M.A.Innis, D.H. Gelfand, J.J. Sninsky & T.J. White Editors.

35 In all constructs, the "outside" primers used are shown in Table 8 and the "overlapping" primers are shown in Table 9.

TABLE 8  
Outside Primers

<b>Cla.FL.F2:</b> 5'ATC GAT ATC GAT AGC CAG ACA CCC CGG CCA G3'	(SEQ ID NO: 47)
<b>HMPLL-R:</b> 5'GCT AGC TCT AGA CAG GGA AGG GAG CTG TAC ATG AGA3'	(SEQ ID NO: 48)

5

TABLE 9  
Overlapping Primers

<b>hML-2:</b> MLΔ4.F: 5'CTC CTT GGA ACC CAG GGC AGG ACC 3' MLΔ4.R 5'GGT CCT GCC CTG GGT TCC AAG GAG 3'	(SEQ ID NO: 49) (SEQ ID NO: 50)
<b>hML-3:</b> hMLΔ116+: 5'CTG CTC CGA GGA AAG GAC TTC TGG ATT 3' hMLΔ116-: 5'AAT CCA GAA GTC CTT TCC TCG GAG CAG 3'	(SEQ ID NO: 51) (SEQ ID NO: 52)
<b>hML(R153A, R154A):</b> RR-KO-F: 5'CCC TCT GCG TCG CGG CGG CCC CAC CCA C 3' RR-KO-R: 5'GTG GGT GGG GCC GCC GCG ACG CAG AGG G 3'	(SEQ ID NO: 53) (SEQ ID NO: 54)

All PCR amplifications were performed with cloned Pfu DNA polymerase 10 (Stratagene) using the following conditions: Initial template denaturation was at 94°C for 7 min. followed by 30 cycles of 1 min. at 94°C, 1 min. at 55°C and 1.5 min. at 72°C. The final cycle was allowed to extend for 10 min. at 72°C. The final PCR product was digested with ClaI-XbaI, gel purified and cloned in pRK5tkneo. 293 cells were transfected with the various constructs as described above and the supernatant was 15 assayed using the Ba/F3-mp1 proliferation assay. hML-2 and hML-3 showed no detectable activity in this assay, however the activity of hML(R153A, R154A) was similar to hML indicating that processing at this di-basic site is not required for activity (see Fig. 13).

**EXAMPLE 12*****Murine mpl Ligand cDNA***  
***mML, mML-2 and mML-3****Isolation of mML cDNA.*

5        A DNA fragment corresponding to the entire coding region of the human *mpl* ligand was obtained by PCR, gel purified and labeled by random priming in the presence of  $^{32}\text{P}$ -dATP and  $^{32}\text{P}$ -dCTP. This probe was used to screen  $10^6$  clones of a mouse liver cDNA library in  $\lambda$ GT10 (Clontech cat# ML3001a). Duplicate filters were hybridized in 35% formamide, 5xSSC, 10xDenhardt's, 0.1% SDS, 0.05M 10      sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 100  $\mu\text{g}/\text{ml}$  of sonicated salmon sperm DNA overnight in the presence of the probe. Filters were rinsed in 2xSSC and then washed once in 0.5xSSC, 0.1% SDS at 42°C. Hybridizing phage were plaque-purified and the cDNA inserts were subcloned into the Eco R1 site of Bluescript SK- plasmid. Clone "LD" with a 1.5 kb insert was chosen for further analysis and both 15      strands were sequenced as described above for the human ML cDNA. The nucleotide and deduced amino acid sequences from clone LD are provided in Fig. 14 (SEQ ID NOS: 1 & 11). The deduced mature ML sequence from this clone was 331 amino acid residues long and identified as mML331 (or mML-2 for reasons described below). Considerable identity for both nucleotide and deduced amino acid sequences were 20      observed in the EPO-like domains of these ML's. However, when deduced amino acid sequences of human and mouse ML's were aligned, the mouse sequence appeared to have a tetrapeptide deletion between human residues 111-114 corresponding to the 12 nucleotide deletion following nucleotide position 618 seen in both the human (see above) and pig (see below) cDNA's. Accordingly, additional clones were examined to 25      detect possible murine ML isoforms. One clone, "L7", had a 1.4 kb insert with a 335 amino acid deduced sequence containing the "missing" tetrapeptide LPLQ. This form is believed to be the full length murine ML and is referred to as mML or mML335. The nucleotide and deduced amino acid sequence for mML are provided in Fig. 16 (SEQ ID NOS: 12 & 13). Finally, clone "L2" was isolated and sequenced. This clone has the 30      116 nucleotide deletion corresponding to hML3 and is therefore denominated mML-3. Comparison of the deduced amino acid sequences of these two isoforms is shown in Fig. 16.

35        *Expression of recombinant mML.* Expression vectors for murine ML were prepared essentially as described in Example 8. Clones encoding mML and mML-2 were subcloned into pRK5tkneo, a mammalian expression vector that provides expression under the control of the CMV promoter and an SV40 polyadenylation signal. The resulting expression vectors, mMLpRKtkneo and mML2pRKtkneo were transiently transfected into 293 cells using the calcium phosphate method. Following transient

transfecti n, m dia was conditioned for five days. The cells wer maintained in high glucose DMEM m dia supplemented with 10% fetal calf serum.

*Expression of murine-*mpl* (mmpl) in Ba/F3 cells.* Stable cell lines expressing c-*mpl* were obtained by transfection of *mmpl* pRKtkneo, essentially as described for human *mpl* in **Example 1**. Briefly, an expression vector (20 µg; linearized) containing the entire coding sequence of murine *mpl* (Skoda, R. C., et al., *EMBO J.* 12:2645-2653 [1993]) was transfected into Ba/F3 cells by electroporation (5 X 10<sup>6</sup> cells, 250 volts, 960 µF) followed by selection for neomycin resistance with 2 mg/ml G418. Expression of *mpl* was assessed by flow cytometry analysis using rabbit anti-murine *mpl*-IgG antisera. Ba/F3 cells were maintained in RPMI 1640 media from WEHI -3B cells as a source of IL-3. Supernatants from 293 cells transiently transfected with both mML and mML-2 were assayed in BaF3 cells transfected with both *mmpl* and *hmp* as described in **Example 1**.

15

#### EXAMPLE 13

##### *Porcine *mpl* Ligand cDNA* *pML and pML-2*

Porcine ML (pML) cDNA was isolated by RACE PCR. Briefly, an oligo dT primer and 2 specific primers were designed based on the sequence of the exon of the porcine ML gene encoding the amino terminus of the ML purified from the aplastic pig serum. cDNA prepared from various aplastic pig tissues was obtained and amplified. A PCR cDNA product of 1342 bp was found in kidney and subcloned. Several clones were sequenced and found to encode the mature pig *mpl* ligand (not including a complete secretion signal). The cDNA was found to encode a 332 amino acid mature protein (pML332) having the sequence shown in Fig. 18 (SEQ ID NOS: 9 & 16).

##### *Method:*

*Isolation of pML gene and cDNA.* Genomic clones of the porcine ML gene were isolated by screening a pig genomic library in EMBL3 (Clontech Inc.) with pR45. The library was screened essentially as described in **Example 7**. Several clones were isolated and the exon encoding amino acid sequence identical to that obtained from the purified ML was sequenced. Porcine ML cDNA were obtained using a modification of the RACE PCR protocol. Two specific ML primers were designed based on the sequence of the pig ML gene. Polyadenylated mRNA was isolated from the kidney of aplastic pigs essentially as previously described. cDNA was prepared by reverse transcription with the BamdT primer

(BamdT: 5' GACTCGAGGGATCCATCGATTTTTTTTTTTTT 3')

(SEQ ID NO: 55)

directed against the polyadenosine tail of the mRNA. An initial round of PCR amplification (28 cycles of 95°C for 60 seconds, 58°C for 60 seconds, and 72°C for ninety seconds) was conducted using the ML specific h-forward-1 primer

(h-forward-1: 5' GCTAGCTCTAGAAATTGCTCCTCGTGGTCATGCTTCT 3')

5 (SEQ ID NO: 43)

and the BAMAD primer

(BAMAD: 5' GACTCGAGGGATCCATCG 3')

(SEQ ID NO: 56)

10 in a 100 ml reaction (50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris pH 8.0, 0.2 mM dNTPs, with 0.05 U/ml AmpliTaq polymerase [Perkin Elmer Inc.]) The PCR product was then digested with Cla1, extracted with phenol-chloroform (1:1), ethanol precipitated, and ligated to 0.1 mg of Bluescript SK- vector (Stratagene Inc.) that had been cut with Cla1 and Kpn 1. After incubation for two hours at room temperature, one fourth of the ligation mixture was added directly to a second round of PCR (22

15 cycles as described above) using a second ML specific forward-1 primer

(forward-1: 5' GCTAGCTCTAGAAGCCGGCTCCTGCCTG 3')

(SEQ ID NO: 57)

and T3-21 (an oligonucleotide that binds to a sequence adjacent to the multiple cloning region within the Bluescript SK- vector):

20 (5' CGAAATTAACCCTCACTAAAG 3')  
(SEQ ID NO: 58).

The resulting PCR product was digested with Xba1 and Cla1 and subcloned into Bluescript SK-. Several clones from independent PCR reactions were sequenced.

Again, a second form, designated pML-2, encoding a protein with a 4 amino acid residue deletion (328 amino acid residues) was identified (see Fig. 21 [SEQ ID NO: 21]). Comparison of pML and pML-2 amino acid sequences shows the latter form is identical except that the tetrapeptide QLPP corresponding to residues 111-114 inclusive have been deleted (see Fig. 22 [SEQ ID NOS: 18 & 21]). The four amino acid deletions observed in murine, human and porcine ML cDNA occur at precisely the

30 same position within the predicted proteins.

#### EXAMPLE 14

##### *CMK Assay for Thrombopoietin (TPO) Induction of Platelet Antigen GPIIb/IIIa Expression*

35 CMK cells are maintained in RMPI 1640 medium (Sigma) supplemented with 10% fetal bovine serum and 10mM glutamine. In preparation for the assay, the cells are harvested, washed and resuspended at 5x10<sup>5</sup> cells/ml in serum-free G1F medium supplemented with 5mg/l bovine insulin, 10mg/l apo-transferrin, 1 X trace

elements. In a 96-well flat-bottom plate, the TPO standard or experimental samples are added to each well at appropriate dilutions in 100  $\mu$ l volumes. 100  $\mu$ l of the CMK cell suspension is added to each well and the plates are incubated at 37°C, in a 5% CO<sub>2</sub> incubator for 48 hours. After incubation, the plates are spun at 1000rpm at 4°C for 5 minutes. Supernatants are discarded and 100  $\mu$ l of the FITC-conjugated GPIIb/IIIa monoclonal 2D2 antibody is added to each well. Following incubation at 4°C for 1 hour, plates are spun again at 1000rpm for five minutes. The supernatants containing unbound antibody are discarded and 200  $\mu$ l of 0.1% BSA-PBS wash is added to each well. The 0.1% BSA-PBS wash step is repeated three times. Cells are then analyzed 10 on a FASCAN using standard one parameter analysis measuring relative fluorescence intensity.

#### EXAMPLE 15

##### *DAMI Assay for Thrombopoletin (TPO) by Measuring Endomitotic Activity of DAMI Cells on 96-well Microtiter Plates*

DAMI cells are maintained in IMDM + 10% horse serum (Gibco) supplemented with 10mM glutamine, 100ng/ml Penicillin G, and 50  $\mu$ g/ml streptomycin. In preparation for the assay, the cells are harvested, washed, and resuspended at 1x10<sup>6</sup>cells/ml in IMDM + 1% horse serum. In a 96-well round-bottom plate, 100  $\mu$ l of the TPO standard or experimental samples is added to DAMI cell suspension. Cells are then incubated for 48 hours at 37°C in a 5% CO<sub>2</sub> incubator. After incubation, plates are spun in a Sorvall 6000B centrifuge at 1000rpm for five minutes at 4°C. Supernatants are discarded and 200  $\mu$ l of PBS-0.1% BSA wash step is repeated. Cells are fixed by the addition of 200  $\mu$ l ice-cold 70% Ethanol-PBS and resuspended by 25 aspiration. After incubation at 4°C for 15 minutes, the plates are spun at 2000 rpm for five minutes and 150  $\mu$ l of 1mg/ml RNase containing 0.1mg/ml propidium iodide and 0.05% Tween-20 is added to each well. Following a one hour incubation at 37°C the changes in DNA content are measured by flow cytometry. Polyploidy is measured and quantitated as follows:

30

Normalized Polyploid Ratio (NPR) =  $\frac{(\% \text{Cells in } >\text{G2+M}/\% \text{Cells in } <\text{G2+M}) \text{ with TPO}}{(\% \text{Cells in } >\text{G2+M}/\% \text{Cells in } <\text{G2+M}) \text{ in control}}$

**EXAMPLE 16*****Thrombopoletin (TPO) In Vivo Assay***  
***(Mouse Platelet Rebound Assay)******In Vivo Assay for  $^{35}$ S Determination of Platelet Production***

5 C57BL6 mice (obtained from Charles River) are injected intraperitoneally (IP) with 1 ml goat anti-mouse platelet serum (6 amps) on day 1 to produce thrombocytopenia. On days 5 and 6, mice are given two IP injections of the factor or PBS as the control. On day 7, thirty  $\mu$ Cl of  $\text{Na}_2^{35}\text{SO}_4$  in 0.1 ml saline are injected intravenously and the percent  $^{35}\text{S}$  incorporation of the injected dose into circulating 10 platelets is measured in blood samples obtained from treated and control mice. Platelet counts and leukocyte counts are made at the same time from blood obtained from the retro-orbital sinus.

**EXAMPLE 17**

15 ***KIRA ELISA for Thrombopoletin (TPO)***  
***by Measuring Phosphorylation of the *mpl-Rse.gD* Chimeric Receptor***

The human *mpl* receptor has been disclosed by Vigon *et al.*, *PNAS, USA* 89:5640-5644 (1992). A chimeric receptor comprising the extracellular domain (ECD) of the *mpl* receptor and the transmembrane (TM) and intracellular domain (ICD) of *Rse* (Mark *et al.*, *J. of Biol. Chem.* 269(14):10720-10728 [1994]) with a carboxyl-terminal flag polypeptide (*i.e.* *Rse.gD*) was made for use in the KIRA ELISA described herein. See Fig. 30 and 31 for a diagrammatic description of the assay.

***(a) Capture agent preparation***

25 Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky *et al.*, *Protein Engineering* 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20° C.

***(b) Anti-phosphotyrosine antibody preparation***

30 Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from UBI (Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

***(c) Ligand***

The *mpl* ligand was prepared by the recombinant techniques described herein. The purified *mpl* ligand was stored at 4 °C. as a stock solution.

***(d) Preparation of *Rse.gD* nucleic acid***

35 Synthetic double stranded oligonucleotides were used to reconstitute the coding sequence for the C-terminal 10 amino acids (880 - 890) of human *Rse* and add an

additional 21 amino acids containing an epitope for the antibody 5B6 and a stop codon.

Table 10 presents the final sequence of the synthetic portion of the fusion gene.

TABLE 10

## 5 Synthetic Double Stranded Portion of Human Rse Fusion Gene

coding strand:	
5'-TGCAGCAAGGGCTACTGCCACACTCGAGCTGCGCAGATGCTAGCCTCAAGA TGGCTG ATCCAAATCGATTCCGGCAAAGATCTTCCGGTCTGTAGAAGCT-3'	(SEQ ID NO: 59)
noncoding (anti-sense) strand:	
5'-AGCTTCTACAGGACCGGAAGATCTTGCCGGAAATCGATTGGATCAGCCA TCTTG AGGCTAGCATCTGCGCAGCTCGAGTGTGGCAGTAGCCCTGCTGCA-3'	(SEQ ID NO: 60)

The synthetic DNA was ligated with the cDNA encoding amino acids 1-880 of human Rse at the PstI site beginning at nucleotide 2644 of the published human Rse cDNA sequence (Mark *et al.*, *Journal of Biological Chemistry* 269(14):10720-10728 [1994]) and HindIII sites in the polylinker of the expression vector

10 pSVI7.ID.LL (See Fig. 32 A-L; SEQ ID NO: 22) to create the expression plasmid pSV.ID.Rse.gD. Briefly, the expression plasmid comprises a dicistronic primary transcript which contains sequence encoding DHFR bounded by 5' splice donor and 3' splice acceptor intron splice sites, followed by sequence that encodes the Rse.gD. The full length (non-spliced) message contains DHFR as the first open reading frame and

15 therefore generates DHFR protein to allow selection of stable transformants.

(e) Preparation of *mpl-Rse.gD* nucleic acid

The expression plasmid pSV.ID.Rse.gD produced as described above was modified to produce plasmid pSV.ID.M.tmRd6 which contained the coding sequences of the ECD of

20 human *mpl* (amino acids 1-491) fused to the transmembrane domain and intracellular domain of Rse.gD (amino acids 429-911). Synthetic oligonucleotides were used to join the coding sequence of a portion of the extracellular domain of human *mpl* to a portion of the Rse coding sequence in a two step PCR cloning reaction as described by Mark *et al.*, *J. Biol. Chem.* 267:26166-26171 (1992). Primers used for the first PCR reaction were M1

25 (5'-TCTCGCTACCGTTACAG-3')  
(SEQ ID NO: 61)

and M2

(5'-CAGGTACCCACCAGGCGGTCTCGGT-3')

(SEQ ID NO: 62)

with a *mpl* cDNA template and R1

(5'-GGGCCATGACACTGTCAA-3')

(SEQ ID NO: 63)

and R2

(5'-GACCGCCACCGAGACCGCCTGGTGGGTACCTGTGGTCCTT-3')

(SEQ ID NO: 64)

with a Rse cDNA template. The Pvull-SmaI portion of this fusion junction was used for  
10 the construction of the full-length chimeric receptor.

(f) *Cell transformation*

DP12.CHO cells (EP 307,247 published 15 March 1989) were electroporated with pSV.ID.M.tmRd6 which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and  
15 was resuspended in 20 $\mu$ l 1/10 Tris EDTA. Then, 10 $\mu$ g of DNA was incubated with 10<sup>7</sup> CHO DP12 cells in 1 ml of PBS on ice for 10 min. before electroporation at 400 volts and 330 $\mu$ f. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

20 (g) *Selection of transformed cells for use in the KIRA ELISA*

Clones expressing *MPL/Rse.gD* were identified by western-blotting of whole cell lysates post-fractionation by SDS-PAGE using the antibody 5B6 which detects the gD epitope tag.

(h) *Media*

25 Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(i) *KIRA ELISA*

30 *Mpl-Rse.gD* transformed DP12.CHO cells were seeded (3x10<sup>4</sup> per well) in the wells of a flat-bottom-96 well culture plate in 100  $\mu$ l media and cultured overnight at 37 °C in 5% CO<sub>2</sub>. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50 $\mu$ l of media containing either experimental samples or 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml *mpl* ligand was then added to each well. The cells were stimulated at 37°C for 30 min., the  
35 well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the chimeric receptors, 100  $\mu$ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml

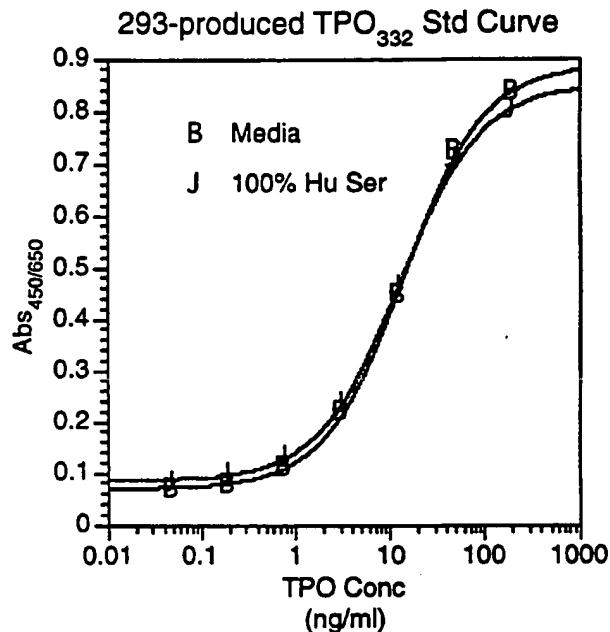
5 aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50  $\mu$ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

10 While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (5.0  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, 100  $\mu$ l/well) was decanted, tamped on a paper towel and blocked with 150  $\mu$ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

15 The lysate containing solubilized MPL/Rse.gD from the cell-culture microtiter well was transferred (85  $\mu$ l/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound *mpl*-Rse.gD was removed by washing with wash buffer and 100  $\mu$ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:18000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), *i.e.* 56 ng/ml was added to each well. After incubation for 2 hr at room temperature the plate was washed and 100  $\mu$ l of horseradish peroxidase (HRPO)-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:60000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100  $\mu$ l freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100  $\mu$ l/well 1.0 M H<sub>3</sub>PO<sub>4</sub>. The absorbance at 450 nm was read with a reference wavelength of 650 nm (ABS450/650), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

20 The standard curve was generated by stimulating dp12.trkA,B or C.gD cells with 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml *mpl* ligand and presented as 25 ng/ml TPO vs. mean ABS450/650  $\pm$  sd using the DeltaSoft program. Sample concentrations were obtained by interpolation of their absorbance on the standard 30 curve and are expressed in terms of ng/ml TPO activity.

The *mpl*-ligand was found to be able to activate the *mpl*-Rse.gD chimeric receptor in a concentration-dependent and ligand-specific manner. Further, the *mpl*-Rse.gD KIRA-ELISA was found to be tolerant of up to 100% human serum (shown) or 100% plasma (not shown), allowing the assay to be used to readily screen patient and 5 pK samples.



10

#### Summary of TPO EC50's

TPO Form (cells)	EC50 (wt/vol)	EC50 (molarity)
Hu TPO 332 (293)	2.56 ng/ml	67.4 pM
Mu TPO 332 (293)	3.69 ng/ml	97.1 pM
Hu TPO 153 (293)	~41 ng/ml	~1.08 nM
Hu TPO 155 ( <i>E. coli</i> )	0.44 ng/ml	11.6 pM
Hu TPO 153met ( <i>E. coli</i> )	0.829 ng/ml	21.8 pM

## EXAMPLE 18

*Receptor Based ELISA for Thrombopoletin (TPO)*

ELISA plates were coated with rabbit F(ab')<sub>2</sub> anti-human IgG (Fc) in pH 9.6 carbonate buffer at 4°C overnight. Plates were blocked with 0.5 % bovine serum albumin in PBS at room temperature for one hour. Fermenter harvest containing the chimeric receptor, *mpI*-IgG, was added to the plates and incubated for 2 hours. Twofold serial dilutions (0.39-25 ng/ml) of the standard (TPO332 produced in 293 cells with the concentration determined by quantitative amino acid analysis) and serially diluted samples in 0.5% bovine serum albumin, 0.05% tween 20 were added to the plates and incubated for 2 hours. Bound TPO was detected with protein A purified, biotinylated rabbit antibodies to TPO155 which was produced in *E. coli* (1 hour incubation), followed by streptavidin-peroxidase (30 min. incubation) and 3,3',5,5'-tetramethyl benzidine as the substrate. The absorbance was read at 450 nm. Plates were washed between steps. For data analysis, the standard curve is fitted using a four-parameter curve fitting program by Kaleidagraph. Concentrations of the samples were calculated from the standard curve.

## EXAMPLE 19

*Expression and Purification of TPO from 293 Cells*20 1. *Preperation of 293 Cell Expression Vectors*

A cDNA corresponding to the TPO entire open reading frame was obtained by PCR using the following oligonucleotides as primers:

TABLE 11

293 PCR Primers

Cla.FL.F: 5' ATC GAT ATC GAT CAG CCA GAC ACC CCG GCC AG 3'	(SEQ ID NO: 65)
hmpI-R: 5' GCT AGC TCT AGA CAG GGA AGG GAG CTG TAC ATG AGA 3'	(SEQ ID NO: 48)

25 PRK5-*hmpI* I (described in Example 9) was used as template for the reaction in the presence of pfu DNA polymerase (Stratagene). Initial denaturation was for 7 min. at 94°C followed by 25 cycles of amplification (1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C). Final extension was for 15 min. at 72°C). The PCR product was purified and cloned between the restriction sites *Cla*I and *Xba*I of the plasmid 30 pRK5tkneo, a pRK5 derived vector modified to express a neomycin resistance gene under the control of the thymidine kinase promoter, to obtain the vector pRK5tkneo.ORF. A second construct corresponding to the *epo* homologous domain was

generated the same way but using Cla.FL.F as forward primer and the following reverse primer:

Arg.STOP.Xba: 5' TCT AGA TCT AGA TCA CCT GAC GCA GAG GGT GGA CC 3'

(SEQ ID NO: 66)

5 The final construct is called pRK5-tkneoEPO-D. The sequence of both constructs was verified as described in **Example 7**.

2. *Transfection of Human Embryonic Kidney cells*

These 2 constructs were transfected into Human Embryonic Kidney cells by the CaPO<sub>4</sub> method as described in **Example 9**. 24 hours after transfection selection of 10 neomycin resistant clones was started in the presence of 0.4 mg/ml G418. 10 to 15 days later individual colonies were transferred to 96 well plates and allowed to grow to confluence. Expression of ML153 or ML332 in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay (described in **Example 1**).

15 3. *Purification of rhML332*

293-rhML332 conditioned media was applied to a Blue-Sepharose (pharmacia) column that was equilibrated in 10mM sodium phosphate pH 7.4 (buffer A). The column was subsequently washed with 10 column volumes each of buffer A and buffer A containing 2M urea. The column was then eluted with buffer A containing 2M urea and 1M NaCl. The Blue-Sepharose elution pool was then directly applied to a WGA-Sepharose column equilibrated in buffer A. The WGA-Sepharose column was then washed with 10 column volumes of buffer A containing 2M urea and 1 M NaCl and eluted with the same buffer containing 0.5M N-acetyl-D-glucosamine. The WGA-Sepharose eluate was applied to a C4-HPLC column (Synchrom, Inc.) equilibrated in 25 0.1% TFA. The C4-HPLC column was eluted with discontinuous propanol gradient (0-25%, 25-35%, 35-70%). rhML332 was found to elute in the 28-30% propanol region of the gradient. By SDS-PAGE the purified rhML332 migrates as a broad band in the 68-80 kDa region of the gel(see **Figure 15**).

4. *Purification of rhML153*

30 293-rhML153 conditioned media was resolved on Blue-Sepharose as described for rhML332. The Blue Sepharose eluate was applied directly to a *mpl*-affinity column as described above. RhML153 eluted from the *mpl*-affinity column was purified to homogeneity using a C4-HPLC column run under the same conditions as described for rhML332. By SDS-PAGE the purified rhML153 resolves into 2 major 35 and 2 minor bands with Mr of ~18,000-21,000(see **Figure 15**).

## EXAMPLE 20

*Expression and Purification of TPO from CHO*1. *Description of CHO Expression Vectors*

The expression vectors used in the electroporation protocols described below  
5 have been designated:

pSVI5.ID.LL.MLORF (full length or hTPO332), and  
pSVI5.ID.LL.MLEPO-D (truncated or hTPO153).

The pertinent features of these plasmids are presented in Fig. 23 and 24.

2. *Preparation of CHO Expression Vectors*

10 A cDNA corresponding to the hTPO entire open reading frame was obtained by PCR using the oligonucleotide primers of Table 12.

TABLE 12

## CHO Expression Vector PCR Primers

Cla.FLF2	5' ATC GAT ATC GAT AGC CAG ACA CCC CGG CCA G 3'	(SEQ ID NO: 47)
ORF.Sal	5' AGT CGA CGT CGA CGT CGG CAG TGT CTG AGA ACC 3'	(SEQ ID NO: 67)

15 PRK5-hmp1 (described in Example 7 and 9) was used as template for the reaction in the presence of pfu DNA polymerase (Stratagene). Initial denaturation was for 7 min. at 94°C followed by 25 cycles of amplification (1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C). Final extension was for 15 min. at 72°C). The PCR product was purified and cloned between the restriction sites ClaI and SalI of the  
20 plasmid pSVI5.ID.LL to obtain the vector pSVI5.ID.LL.MLORF. A second construct corresponding to the EPO homologous domain was generated the same way but using Cla.FLF2 as forward primer and the following reverse primer:

EPOD.Sal 5' AGT CGA CGT CGA CTC ACC TGA CGC AGA GGG TGG ACC 3'  
(SEQ ID NO: 68)

25 The final construct is called pSVI5.ID.LL.MLEPO-D. The sequence of both constructs was verified as described in Example 7 and 9.

In essence, the coding sequences for the full length and truncated ligand were introduced into the multiple cloning site of the CHO expression vector pSVI5.ID.LL. This vector contains the SV40 early promoter/enhancer region, a modified splice unit  
30 containing the mouse DHFR cDNA, a multiple cloning site for the introduction of the gene of interest (in this case the TPO sequences described) an SV40 polyadenylation signal and origin of replication and the beta-lactamase gene for plasmid selection and amplification in bacteria.

3. *Methodology for Establishing Stable CHO Cell Lines Expressing Recombinant Human TPO332 and TPO153*

*a. Description of CHO parent cell line*

The host CHO (Chinese Hamster Ovary) cell line used for the expression of the 5 TPO molecules described herein is known as CHO-DP12 (see EP 307,247 published 15 March 1989). This mammalian cell line was clonally selected from a transfection of the parent line (CHO-K1 DUX-B11(DHFR-)- obtained from Dr. Frank Lee of Stanford University with the permission of Dr.L. Chasin) with a vector expressing preproinsulin to obtain clones with reduced insulin requirements. These cells are also 10 DHFR minus and clones can be selected for the presence of DHFR cDNA vector sequences by growth on medium devoid of nucleoside supplements (glycine, hypoxanthine, and thymidine). This selection system for stably expressing CHO cell lines is commonly used.

*b. Transfection method (electroporation)*

15 TPO332 and TPO153 expressing cell lines were generated by transfecting DP12 cells via electroporation (see e.g. Andreason, G.L. *J. Tiss. Cult. Meth.*, 15,56 [1993]) with linearized pSV15.ID.LL.MLORF or pSV15.ID.LL.MLEPO-D plasmids respectively. Three (3) restriction enzyme reaction mixtures were set up for each 20 plasmid cutting; 10 $\mu$ g, 25 $\mu$ g and 50 $\mu$ g of the vector with the enzyme NOTI by standard molecular biology methods. This restriction site is found only once in the vector in the linearization region 3' and outside the TPO ligand transcription units (see Fig. 23). The 100 $\mu$ l reactions were set up for overnight incubation at 37 degrees. The next day 25 the mixes were phenol-chloroform-isoamyl alcohol (50:49:1) extracted one time and ethanol precipitated on dry ice for approximately one hour. The precipitate was then collected by a 15 minute microcentrifugation and dried. The linearized DNA was resuspended into 50 $\mu$ l of Ham's DMEM-F12 1:1 medium supplemented with standard antibiotics and 2mM glutamine.

Suspension growing DP12 cells were collected, washed one time in the medium 30 described for resuspending the DNA and finally resuspended in the same medium at a concentration of 10<sup>7</sup> cells per 750 $\mu$ l. Aliquots of cells (750 $\mu$ l) and each linearized DNA mix were incubated together at room temperature for one hour and then transferred to a BRL electroporation chamber. Each reaction mix was then 35 electroporated in a standard BRL electroporation apparatus at 350 volts set at 330 $\mu$ F and low capacitance. After electroporation, the cells were allowed to sit in the apparatus for 5 minutes and then on ice for an additional 10 minute incubation period. The electroporated cells were transferred to 60mm cell culture dishes containing 5 ml of standard, complete growth medium for CHO cells (High glucose DMEM-F12 50:50

without glycine supplemented with 1X GHT, 2mM glutamine, and 5% fetal calf serum) and grown overnight in a 5% CO<sub>2</sub> cell culture incubator.

*c. Selection and screening method*

The next day, cells were trypsinized off the plates by standard methods and 5 transferred to 150mm tissue culture dishes containing DHFR selective medium (Ham's DMEM-F12, 1:1 medium described above supplemented with either 2% or 5% dialyzed fetal calf serum but devoid of glycine, hypoxanthine and thymidine this is the standard DHFR selection medium we use). Cells from each 60mm dish were subsequently replated into 5 /150 mm dishes. Cells were then incubated for 10 to 15 days( with 10 one medium change) at 37 degrees/5% CO<sub>2</sub> until clones began to appear and reached sizes amenable to transfer to 96 well dishes. Over a period of 4-5 days, cell lines 15 were transferred to 96 well dishes using sterile yellow tips on a pipettman set at 50ml. The cells were allowed to grow to confluence (usually 3-5 days) and then the trays were trypsinized and 2 copies of the original tray were reproduced. Two of these 20 copies were short term stored in the freezer with cells in each well diluted into 50 $\mu$ l of 10%FCS in DMSO. 5 day conditioned serum free medium samples were assayed from confluent wells in the third tray for TPO expression via the Ba/F cell based activity assay. The highest expressing clones based on this assay were revived from storage and scaled up to 2 confluent 150mm T-flasks for transfer to the cell culture group for 25 suspension adaptation, re-assay and banking.

*d. Amplification Protocol*

Several of the highest titer cell lines from the selection described above were subsequently put through a standard methotrexate amplification regime to generate higher titer clones. CHO cell clones are expanded and plated in 10cm dishes at 4 25 concentrations of methotrexate (i.e.. 50nM, 100nM, 200nM and 400nM) at two or three cell numbers (105, 5x105, and 106 cells per dish). These cultures are then incubated at 37 degree/5% CO<sub>2</sub> until clones are established and amenable to transfer to 96 well dishes for further assay. Several high titer clones from this selection were again subjected to greater concentrations of methotrexate (i.e. 600nM, 800 nM, 30 1000nM and 1200nM) and as before resistant clones are allowed to establish and then transferred to 96 well dishes and assayed.

**4. Culturing Stable CHO Cell Lines Expressing Recombinant Human TPO<sub>332</sub> and TPO<sub>153</sub>**

Banked cells are thawed and the cell population is expanded by standard cell 35 growth methods in either serum free or serum containing medium. After expansion to sufficient cell density, cells are washed to remove spent cell culture media. Cells are then cultured by any standard method including; batch, fed-batch or continuous culture at 25-40 °C, neutral pH, with a dissolved O<sub>2</sub> content of at least 5% until the

constitutively secreted TPO is accumulated. Cell culture fluid is then separated from the cells by mechanical means such as centrifugation.

5      *Purification of Recombinant Human TPO from CHO Culture Fluids*

Harvested cell culture fluid (HCCF) is directly applied to a Blue Sepharose 6  
5 Fast Flow column (Pharmacia) equilibrated in 0.01M Na Phosphate pH7.4, 0.15M NaCl  
at a ratio of approximately 100L of HCCF per liter of resin and at a linear flow rate of  
approximately 300 ml/hr/cm<sup>2</sup>. The column is then washed with 3 to 5 column  
volumes of equilibration buffer followed by 3 to 5 column volumes of 0.01M Na  
Phosphate pH7.4, 2.0M urea. The TPO is then eluted with 3 to 5 column volumes of  
10 0.01M Na Phosphate pH7.4, 2.0M urea, 1.0M NaCl.

The Blue Sepharose Pool containing TPO is then applied to a Wheat Germ Lectin  
Sepharose 6MB column (Pharmacia) equilibrated in 0.01M Na Phosphate pH7.4,  
2.0M urea, and 1.0M NaCl at a ratio of from 8 to 16 ml of Blue Sepharose Pool per ml  
of resin at flow rate of approximately 50 ml/hr/cm<sup>2</sup>. The column is then washed with  
15 2 to 3 column volumes of equilibration buffer. The TPO is then eluted with 2 to 5  
column volumes of 0.01M Na Phosphate pH7.4, 2.0M urea, 0.5M N-acetyl-D-  
glucosamine.

The Wheat Germ Lectin Pool is then adjusted to a final concentration of 0.04%  
C<sub>12</sub>E<sub>8</sub> and 0.1% trifluoroacetic acid (TFA). The resulting pool is applied to a C4  
20 reverse phase column (Vydac 214TP1022) equilibrated in 0.1% TFA, 0.04% C<sub>12</sub>E<sub>8</sub>  
at a load of approximately 0.2 to 0.5 mg protein per ml of resin at a flow rate of 157  
ml/hr/cm<sup>2</sup>.

The protein is eluted in a two phase linear gradient of acetonitrile containing  
0.1% TFA, 0.04% C<sub>12</sub>E<sub>8</sub>. The first phase is composed of a linear gradient from 0 to  
25 30% acetonitrile in 15 minutes, The second phase is composed of a linear gradient  
from 30 to 60% acetonitrile in 60 minutes. The TPO elutes at approximately 50%  
acetonitrile. A pool is made on the basis of SDS-PAGE.

The C4 Pool is then diluted with 2 volumes of 0.01M Na Phosphate pH7.4,  
0.15M NaCl and diafiltrated versus approximately 6 volumes of 0.01M Na Phosphate  
30 pH7.4, 0.15M NaCl on an Amicon YM or like ultrafiltration membrane having a  
10,000 to 30,000 Dalton molecular weight cut-off. The resulting diafiltrate may be  
then directly processed or further concentrated by ultrafiltration. The  
diafiltrate/concentrate is adjusted to a final concentration of 0.01% Tween-80.

All or a portion of the diafiltrate/concentrate equivalent to 2 to 5% of the  
35 calculated column volume is then applied to a Sephadryl S-300 HR column  
(Pharmacia) equilibrated in 0.01M Na Phosphate pH7.4, 0.15M NaCl, 0.01% Tween-  
80 and chromatographed at a flow rate of approximately 17 ml/hr/cm<sup>2</sup>. The TPO  
containing fractions which are free of aggregate and proteolytic degradation products

are pooled on the basis of SDS-PAGE. The resulting pool is filtered on a 0.22 $\mu$  filter, Millex-GV or like, and stored at 2-8°C.

#### EXAMPLE 21

5        *Transformation and Induction of TPO Protein Synthesis In E. coli*

1        *Construction of E. coli TPO expression vectors*

The plasmids pMP21, pMP151, pMP41, pMP57 and pMP202 are all designed to express the first 155 amino acids of TPO downstream of a small leader which varies among the different constructs. The leaders provide primarily for high level 10 translation initiation and rapid purification. The plasmids pMP210-1, -8, -21, -22, -24, -25 are designed to express the first 153 amino acids of TPO downstream of an initiation methionine and differ only in the codon usage for the first 6 amino acids of TPO, while the plasmid pMP251 is a derivative of pMP210-1 in which the carboxy 15 terminal end of TPO is extended by two amino acids. All of the above plasmids will produce high levels of intracellular expression of TPO in E. coli upon induction of the tryptophan promoter (Yansura, D. G. et. al. *Methods in Enzymology* ( Goeddel, D. V., Ed.) 185:54-60, Academic Press, San Diego [1990]). The plasmids pMP1 and pMP172 are intermediates in the construction of the above TPO intracellular expression plasmids.

20        (a) *Plasmid pMP1*

The plasmid pMP1 is a secretion vector for the first 155 amino acids of TPO, and was constructed by ligating together 5 fragments of DNA as shown in Fig. 33. The first of these was the vector pPho21 in which the small MluI-BamHI fragment had been removed. pPho21 is a derivative of phGH1 (Chang, C. N. et. al., *Gene* 55:189-25 196 [1987]) in which the human growth hormone gene has been replaced with the E. coli phoA gene, and a MluI restriction site has been engineered into the coding sequence for the STII signal sequence at amino acids 20-21.

The next two fragments, a 258 base pair *Hinf*I-PstI piece of DNA from pRK5-*hmp*/I (Example 9) encoding TPO amino acids 19-103, and the following synthetic 30 DNA encoding amino acids 1-18

5'-CGCGTATGCCAGCCGGCTCCTCCTGCTTGACCTCCGAGTCCTCAGTAAACTGCTTCG  
TG

35        ATACGGTCGGGCCAGGGAGGACGAACACTGGAGGCTCAGGAGTCATTGACGAAGC  
ACTGA-5'

(SEQ ID NO: 69)

(SEQ ID NO: 70)

were preligated with T4-DNA ligase, and second cut with PstI. The fourth was a 152 base pair PstI-HaeIII fragment from pRK5hmpII encoding amino acids 104-155 of TPO. The last was a 412 base pair StuI-BamHI fragment from pdh108 containing the lambda to transcriptional terminator as previously described (Scholtissek, S. et. al., 5 *NAR* 15:3185 [1987]).

5 (b) *Plasmid pMP21*

The plasmid pMP21 is designed to express the first 155 amino acids of TPO with the aid of a 13 amino acid leader comprising part of the STII signal sequence. It was constructed by ligating together three (3) DNA fragments as shown in Fig. 34, 10 the first of these being the vector pVEG31 in which the small XbaI-SphI fragment had been removed. The vector pVEG31 is a derivative of pHGH207-1 (de Boer, H. A. et. al., , in *Promoter Structure and Function* (Rodriguez, R. L. and Chamberlain, M. J. , Ed), 462, Praeger, New York [1982]) in which the human growth hormone gene has been replaced by the gene for vascular endothelial growth factor ( this identical vector 15 fragment can be obtained from this latter plasmid).

The second part in the ligation was a synthetic DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTCTTCTTAA  
20 TTAATACTTTCTTATAGCGTAAAGAAGAATTGCGC-5'  
(SEQ ID NO: 71)  
(SEQ ID NO: 72)

The last piece was a 1072 base pair MluI-SphI fragment from pMP1 encoding 155 amino acids of TPO.

25 (c) *Plasmid pMP151*

The plasmid pMP151 is designed to express the first 155 amino acids of TPO downstream of a leader comprising 7 amino acids of the STII signal sequence, 8 histidines, and a factor Xa cleavage site. As shown in Fig. 35, pMP151 was constructed by ligating together three DNA fragments, the first of these being the 30 previously described vector pVEG31 from which the small XbaI-SphI fragment had been removed. The second was a synthetic DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTCTCACCATCACCATCACATCGAAG  
GTCGTAGCC  
35 TTAATACTTTCTTATAGCGTAAAGTAGTGGTAGTGGTAGTGGTAGTGTAGCTTC  
CAGCAT-5'  
(SEQ ID NO: 73)  
(SEQ ID NO: 74)

The last was a 1064 base pair BglI-SphI fragment from pMP11 encoding 154 amino acids of TPO. The plasmid pMP11 is identical to pMP1 with the exception of a few codon changes in the STII signal sequence (this fragment can be obtained from pMP1).

(d) *Plasmid pMP202*

5 The plasmid pMP202 is very similar to the expression vector pMP151 with the exception that the factor Xa cleavage site in the leader has been replaced with a thrombin cleavage site. As shown in Fig. 36, pMP202 was constructed by ligating together three DNA fragments. The first of these was the previously described pVEG31 in which the small XbaI-SphI fragment had been removed. The second was a synthetic  
10 DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTTCATCACCATCACCATCACATCGAA  
CCACGTAGCC

TTAATACTTTCTTATAGCGTAAAGTAGTGGTAGTGGTAGTGGTAGTGTAGCTT

15 GGTGCAT-5'

(SEQ ID NO: 75)

(SEQ ID NO: 76)

The last piece was a 1064 base pair BglI-SphI fragment from the previously described plasmid pMP11.

20 (e) *Plasmid pMP172*

The plasmid pMP172 is a secretion vector for the first 153 amino acids of TPO, and is an intermediate for the construction of pMP210. As shown in Fig. 37, pMP172 was prepared by ligating together three DNA fragments, the first of which was the vector pLS32lamB in which the small EcoRI-HindIII section had been removed.

25 The second was a 946 base pair EcoRI-Hgal fragment from the previously described plasmid pMP11. The last piece was a synthetic DNA duplex with the following sequence:

5'-TCCACCCCTCTGCGTCAGGT (SEQ ID NO: 77)  
GGAGACGCAGTCCATCGA-5' (SEQ ID NO: 78)

30 (f) *Plasmid pMP210*

The plasmid pMP210 is designed to express the first 153 amino acids of TPO after a translational initiation methionine. This plasmid was actually made as a bank of plasmids in which the first 6 codons of TPO were randomized in the third position of each codon, and was constructed as shown in Fig. 38 by the ligation of three DNA fragments. The first of these was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was a synthetic DNA duplex shown below treated first with DNA polymerasel (Klenow) followed by digestion with

XbaI and HindIII, and encoding the initiation methionine and the randomized first 6 codons of TPO.

5'-GCAGCAGTTCTAGAATTATGTCNCCNGCNCCNCNTGTGACCTCCGA  
5 AACTGGAGGCT  
GTTCTCAGTAAA (SEQ ID NO: 79)  
CAAGAGTCATTTGACGAAGCACTGAGGGTACAGGAAG-5' (SEQ ID NO: 80)

10 The third was a 890 base pair HindIII-SphI fragment from pMP172 encoding amino acids 19-153 of TPO.

15 The plasmid pMP210 bank of approximately 3700 clones was retransformed onto high tetracycline (50 µg/ml) LB plates to select out high translational initiation clones (Yansura, D. G. et. al., *Methods: A Companion to Methods in Enzymology* 4:151-158 [1992]). Of the 8 colonies which came up on high tetracycline plates, five of the best in terms of TPO expression were subject to DNA sequencing and the results are shown in Fig. 39 (SEQ ID NOS: 23, 24, 25, 26, 27 and 28).

(g) *Plasmid pMP41*

20 The plasmid pMP41 is designed to express the first 155 amino acids of TPO fused to a leader consisting of 7 amino acids of the STII signal sequence followed by a factor Xa cleavage site. The plasmid was constructed as shown in Fig. 40 by ligating together three pieces of DNA, the first of which was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was the following synthetic DNA duplex:

25 5'-CTAGAATTATGAAAAAGAATATCGCATTATCGAAGGTCGTAGCC (SEQ ID NO: 81)  
TTAATACTTTTCTTATAGCGTAAATAGCTTCCAGCAT-5' (SEQ ID NO: 82)

The last piece of the ligation was the 1064 base pair BglII-SphI fragment from the previously described plasmid pMP11.

(h) *Plasmid pMP57*

30 The plasmid pMP57 expresses the first 155 amino acids of TPO downstream of a leader consisting of 9 amino acids of the STII signal sequence and the dibasic site Lys-Arg. This dibasic site provides for a means of removing the leader with the protease ArgC. This plasmid was constructed as shown in Fig. 41 by ligating together three DNA pieces. The first of these was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was the following 35 synthetic DNA duplex:

5'-CTAGAATTATGAAAAAGAATATCGCATTCTTCTTAAACGTAGCC (SEQ ID NO: 83)  
TTAATACTTTTCTTATAGCGTAAAGAAGAATTGCAT-5' (SEQ ID NO: 84)

The last part of the ligation was the 1064 base pair BglII-SphI fragment from the previously described plasmid pMP11.

(i) *Plasmid pMP251*

The plasmid pMP251 is a derivative of pMP210-1 in which two additional 5 amino acids of TPO are included on the carboxy terminal end. As shown in Fig.42, this plasmid was constructed by ligating together two pieces of DNA, the first of these being the previously described pMP21 in which the small XbaI-ApaI fragment had been removed. The second part of the ligation was a 316 base pair XbaI-ApaI fragment from pMP210-1.

10 2. *Transformation and Induction of E. coli with TPO expression vectors*

The above TPO expression plasmids were used to transform the *E. coli* strain 44C6 (w3110 tonA<sub>Δ</sub> rpoH<sub>ts</sub> lon<sub>Δ</sub> clpP<sub>Δ</sub> galE) using the CaCl<sub>2</sub> heat shock method (Mandel, M. et al., *J. Mol. Biol.*, 53:159-162, [1970]). The transformed cells were grown first at 37°C in LB media containing 50 µg/ml carbenicillin until the optical 15 density (600nm) of the culture reached approximately 2-3. The LB culture was then diluted 20x into M9 media containing 0.49% casamino acids (w/v) and 50 µg/ml carbenicillin. After growth with aeration at 30°C for 1 hour, indole-3-acrylic acid was added to a final concentration of 50 µg/ml. The culture was then allowed to continue growing at 30°C with aeration for another 15 hours at which time the cells 20 were harvested by centrifugation.

## EXAMPLE 22

### *Production of Biologically Active TPO (Met<sup>1</sup> 1-153) in E. coli*

The procedures given below for production of biologically active, refolded TPO 25 (met<sup>1</sup> 1-153) can be applied in analogy for the recovery of other TPO variants including N and C terminal extended forms (see Example 23).

A *Recovery of non-soluble TPO (Met<sup>1</sup> 1-153)*

*E. coli* cells expressing TPO (Met<sup>1</sup> 1-153) encoded by the plasmid pMP210-1 are fermented as described above. Typically, about 100g of cells are resuspended in 30 1 L (10 volumes) of cell disruption buffer (10 mM Tris, 5 mM EDTA, pH 8) with a Polytron homogenizer and the cells centrifuged at 5000 x g for 30 minutes. The washed cell pellet is again resuspended in 1 L cell disruption buffer with the Polytron homogenizer and the cell suspension is passed through an LH Cell Disrupter (LH Inceltech, Inc.) or through a Microfluidizer (Microfluidics International) according to 35 the manufacturers' instructions. The suspension is centrifuged at 5,000g for 30 min. and resuspended and centrifuged a second time to make a washed refractile body pellet. The washed pellet is used immediately or stored frozen at -70°C.

**B. Solubilization and purification of monomeric TPO (Met<sup>1</sup> 1-153)**

The pellet from above is resuspended in 5 volumes by weight of 20 mM Tris, pH 8, with 6-8 M guanidine and 25 mM DTT (dithiothreitol) and stirred for 1-3 hr., or overnight, at 4°C to effect solubilization of the TPO protein. High concentrations of 5 urea (6-8M) are also useful but generally result in lower yields compared to guanidine. After solubilization, the solution is centrifuged at 30,000 x g for 30 min. to produce a clear supernatant containing denatured, monomeric TPO protein. The supernatant is then chromatographed on a Superdex 200 gel filtration column (Pharmacia, 2.6 x 60 cm) at a flow rate of 2 ml/min. and the protein eluted with 20 10 mM Na phosphate, pH 6.0, with 10 mM DTT. Fractions containing monomeric, denatured TPO protein eluting between 160 and 200 ml are pooled. The TPO protein is further purified on a semi-preparative C4 reversed phase column (2 x 20 cm VYDAC). The sample is applied at 5 ml/min. to a column equilibrated in 0.1% 15 TFA(trifluoroacetic acid) with 30% acetonitrile. The protein is eluted with a linear gradient of acetonitrile (30-60% in 60 min.). The purified reduced protein elutes at approximately 50% acetonitrile. This material is used for refolding to obtain biologically active TPO variant.

**C. Generation of biologically active TPO (Met<sup>1</sup> 1-153)**

Approximately 20 mg of monomeric, reduced and denatured TPO protein in 40 20 ml 0.1% TFA/50% acetonitrile is diluted into 360 ml of refolding buffer containing optimally the following reagents:

50 mM Tris  
0.3 M NaCl  
5 mM EDTA  
25 25% CHAPS detergent  
25% glycerol  
5 mM oxidized glutathione  
1 mM reduced glutathione  
pH adjusted to 8.3  
30 After mixing, the refolding buffer is gently stirred at 4°C for 12-48 hr to effect maximal refolding yields of the correct disulfide-bonded form of TPO (see below). The solution is then acidified with TFA to a final concentration of 0.2%, filtered through a 0.45 or 0.22 micron filter, and 1/10 volume of acetonitrile added. This solution is then pumped directly onto a C4 reversed phase column and the 35 purified, refolded TPO (Met<sup>1</sup> 1-153) eluted with the same gradient program as above. Refolded, biologically active TPO is eluted at approximately 45% acetonitrile under these conditions. Improper disulfide-bonded versions of TPO are eluted earlier.

The final purified TPO (Met<sup>1</sup> 1-153) is greater than 95% pure as assessed by SDS gels and analytical C4 reversed phase chromatography. For animal studies, the C4 purified material was dialyzed into physiologically compatible buffers. Isotonic buffers (10 mM Na acetate, pH 5.5, 10 mM Na succinate, pH 5.5 or 10 mM Na phosphate, pH 7.4) containing 150 mM NaCl and 0.01% Tween 80 were utilized.

Because of the high potency of TPO in the Ba/F3 assay (half maximal stimulation is achieved at approximately 3 pg/ml), it is possible to obtain biologically active material utilizing many different buffer, detergent and redox conditions. However, under most conditions only a small amount of properly folded material (<10%) is obtained. For commercial manufacturing processes, it is desirable to have refolding yields at least 10%, more preferably 30-50% and most preferably >50%. Many different detergents (Triton X-100, dodecyl-beta-maltoside, CHAPS, CHAPSO, SDS, sarkosyl, Tween 20 and Tween 80, Zwittergent 3-14 and others) were assessed for efficiency to support high refolding yields. Of these detergents, only the CHAPS family (CHAPS and CHAPSO) were found to be generally useful in the refolding reaction to limit protein aggregation and improper disulfide formation. Levels of CHAPS greater than 1% were most useful. Sodium chloride was required for best yields, with the optimal levels between 0.1 M and 0.5M. The presence of EDTA (1-5 mM) limited the amount of metal-catalyzed oxidation (and aggregation) which was observed with some preparations. Glycerol concentrations of greater than 15% produced the optimal refolding conditions. For maximum yields, it was essential to have both oxidized and reduced glutathione or oxidized and reduced cysteine as the redox reagent pair. Generally higher yields were observed when the mole ratio of oxidized reagent is equal to or in excess over the reduced reagent member of the redox pair. pH values between 7.5 and about 9 were optimal for refolding of these TPO variants. Organic solvents (e.g. ethanol, acetonitrile, methanol) were tolerated at concentrations of 10-15% or lower. Higher levels of organic solvents increased the amount of improperly folded forms. Tris and phosphate buffers were generally useful. Incubation at 4°C also produced higher levels of properly folded TPO.

Refolding yields of 40-60% (based on the amount of reduced and denatured TPO used in the refolding reaction) are typical for preparations of TPO that have been purified through the first C4 step. Active material can be obtained when less pure preparations (e.g. directly after the Superdex 200 column or after the initial refractile body extraction) although the yields are less due to extensive precipitation and interference of non-TPO proteins during the TPO refolding process.

Since TPO (Met<sup>1</sup> 1-153) contains 4 cysteine residues, it is possible to generate three different disulfide versions of this protein:

version 1: disulfides between cysteine residues 1-4 and 2-3

version 2: disulfides between cysteine residues 1-2 and 3-4

version 3: disulfides between cysteine residues 1-3 and 2-4.

During the initial exploration in determining refolding conditions, several different peaks containing the TPO protein were separated by C4 reversed phase chromatography. Only one of these peaks had significant biological activity as determined using the Ba/F3 assay. Subsequently, the refolding conditions were optimized to yield preferentially that version. Under these conditions, the misfolded versions are less than 10-20% of the total monomer TPO obtained.

The disulfide pattern for the biologically active TPO has been determined to be 1-4 and 2-3 by mass spectrometry and protein sequencing(*i.e.* version 1). Aliquots of the various C4-resolved peaks (5-10 nmoles) were digested with trypsin (1:25 mole ratio of trypsin to protein). The digestion mixture was analyzed by matrix-assisted laser desorption mass spectrometry before and after reduction with DTT. After reduction, masses corresponding to most of the larger tryptic peptides of TPO were detected. In the un-reduced samples, some of these masses were missing and new masses were observed. The mass of the new peaks corresponded basically to the sum of the individual tryptic peptides involved in the disulfide pair. Thus it was possible to unequivocally assign the disulfide pattern of the refolded, recombinant, biologically active TPO to be 1-4 and 2-3. This is consistent with the known disulfide pattern of 20 the related molecule erythropoietin.

*D. Biological activity of recombinant, refolded TPO (met 1-153)*

Refolded and purified TPO (Met<sup>1</sup> 1-153) has activity in both *in vitro* and *in vivo* assays. In the Ba/F3 assay, half-maximal stimulation of thymidine incorporation into the Ba/F3 cells was achieved at 3.3 pg /ml (0.3 pM). In the *mpl* receptor-based 25 ELISA, half-maximal activity occurred at 1.9 ng/ml (120 pM). In normal and myelosuppressed animals produced by near-lethal X-radiation, TPO (Met<sup>1</sup> 1-153) was highly potent (activity was seen at doses as low as 30 ng/mouse) to stimulate the production of new platelets.

30

**EXAMPLE 23**

***Production of Other Biologically Active TPO Variants in E. coli***

Three different TPO variants produced in *E. coli*, purified and refolded into biological active forms are provided below.

(1) MLF - 13 residues from the bacterial-derived signal sequence STII are 35 fused to the N-terminal domain of TPO (residues 1-155). The resulting sequence is

MKKNIAFLLNAYASPAPPAC....CVRRA (SEQ ID NO: 85)

where the leader sequence is underlined and C----C represents Cys<sup>7</sup> through Cys<sup>151</sup>. This variant was constructed to provide a tyrosine for radio-iodination of TPO for receptor and biological studies.

(2) H8MLF - 7 residues from the STII sequence, 8 histidine residues and 5 the Factor Xa enzymatic cleavage sequence IEGR are fused to the N-terminal domain (residues 1-155) of TPO. The sequence is

MKKNIAFHHHHHHHIEGRSPAPPAC----CVRRA (SEQ ID NO: 86)

where the leader sequence is underlined and C----C represents Cys<sup>7</sup> through Cys<sup>151</sup>. This variant, when purified and refolded, can be treated with the enzyme Factor Xa 10 which will cleave after the arginine residue of the sequence IEGR yielding a TPO variant of 155 residues in length with a natural serine N-terminal amino acid.

(3) T-H8MLF - is prepared as described above for variant (2), except a thrombin sensitive sequence IEPR is fused to the N-terminal domain of TPO. The resulting sequence is

15 MKKNIAFHHHHHHHIEPRSPAPPAC----CVRRA (SEQ ID NO: 87)

where the leader sequence is underlined and C----C represents Cys<sup>7</sup> through Cys<sup>151</sup>. This variant, after purification and refolding can be treated with the enzyme thrombin to generate a natural N-terminal variant of TPO of 155 residues in length.

A. Recovery, solubilization and purification of monomeric, biologically active TPO 20 variants (1), (2), and (3).

All of the variants were expressed in E. coli. The majority of the variants were found in refractile bodies, as observed in Example 22 for TPO (Met<sup>-1</sup> 1-153). Identical procedures for the recovery, solubilization and purification of monomeric TPO variants was achieved as described in Example 22. Identical refolding 25 conditions to those used for TPO (Met<sup>-1</sup> 1-153) were used with overall yields of 30-50%. After refolding, the TPO variants were purified by C4 reversed phase chromatography in 0.1% TFA utilizing an acetonitrile gradient as described previously. All of the TPO variants (in their unproteolyzed forms) had biological activity as assessed by the Ba/F3 assay, with half-maximal activities of 2-5 pM.

30 B. Proteolytic processing of Variants (2) and (3) to generate authentic N-terminal TPO (1-155).

TPO variants (2) and (3) above were designed with an enzymatically-cleavable leader peptide before the normal N-terminal amino acid residue of TPO. After refolding and purification of variants (2) and (3) as described above, each was 35 subjected to digestion with the appropriate enzyme. For each variant, the acetonitrile from the C4 reversed phase step was removed by blowing a gentle stream of nitrogen

on the solution. Thereafter the two variants were treated with either Factor Xa or thrombin as described below.

For TPO variant (2), 1 M Tris buffer, pH 8, was added to the acetonitrile-free solution to a final concentration of 50 mM and the pH was adjusted to 8 if necessary.

5 NaCl and CaCl<sub>2</sub> were added to 0.1 M and 2 mM, respectively. Factor Xa (New England Biolabs) was added to achieve about a 1:25 to 1:100 mole ratio of enzyme to variant. The sample was incubated at room temperature for 1-2 hr. to achieve maximal cleavage as assessed by a change in migration on SDS gels representing the loss of the leader sequence. Thereafter, the reaction mixture was purified by C4 reversed phase

10 chromatography using the same gradient and conditions as described above for the purification of properly folded variants. Uncleaved variant B was separated from cleaved variant (2) by these conditions. The N-terminal amino acids were shown to be SPAPP, indicating that removal of the N-terminal leader sequence was successful. Factor Xa also generated variable amounts of an internal cleavage within the TPO

15 domain; cleavage was observed after the arginine residue at position number 118 generating an additional N-terminal sequence of TTAHKDP (SEQ ID NO: 88). On non-reducing SDS gels, a single band at approximately 17000 daltons was observed for the Factor Xa cleaved variant; on reducing gels two bands were seen of molecular weight of approximately 12000 and 5000 daltons, consistent with cleavage at arginine 118.

20 This observation also confirmed that the two parts of the molecule were held together by a disulfide bond between the 1st and 4th cysteine residues, as deduced from the tryptic digestion experiments described above. In the Ba/F3 biological assay, the purified TPO (1-155) variant, after removal of the N-terminal leader sequence and with the internal cleavage, had a half-maximal activity of 0.2 to 0.3 picomolar. The

25 intact variant with the leader sequence had a half-maximal activity of 2-4 picomolar.

For variant (3), the digestion buffer consisted of 50 mM Tris, pH 8, 2% CHAPS, 0.3 M NaCl, 5 mM EDTA and human or bovine thrombin (Calbiochem) at a 1:25 to 1:50 by weight of enzyme to TPO variant protein. Digestion was conducted at room temperature for 2-6 hours. The progress of the digestion was assessed by SDS

30 gels as described above for the Factor Xa cleavage reaction. Generally, more than 90% cleavage of the leader sequence was achieved in this time. The resultant TPO was purified on C4 reversed phase columns as described above and was shown to have the desired N-terminal by amino acid sequencing. Only very minor (<5%) amounts of an internal cleavage at the same arginine-threonine bond as observed above with Factor

35 Xa was obtained. The resultant TPO protein had high biological activity with half-maximal responses in the Ba/F3 assay at 0.2-0.4 picomolar protein. In the *mpl* receptor based ELISA, this protein had a half-maximal response at 2-4 ng/ml purified protein (120-240 picomolar) while the intact variant containing the leader sequence

was less potent in both assays by 5-10 fold. For animal studies, the HPLC-purified cleaved protein was dialyzed into physiological acceptable buffers, with 150 mM NaCl, 0.01% Tween 80 and 10 mM sodium succinate, pH 5.5, or 10 mM sodium acetate, pH 5.5, or 10 mM sodium phosphate, pH 7.4. By HPLC and SDS gels, the purified protein 5 was stable for several weeks when stored at 4°C. In normal and myelosuppressed mice, this purified TPO with the authentic N-terminal sequence was highly active, stimulating the production of platelets at doses as low as 30 ng/mouse.

#### EXAMPLE 24

##### 10 *Synthetic mpl Ligand*

Although Human *mpl* ligand (hML) is usually made using recombinant methods, it can also be synthesized via enzymatic ligation of synthetic peptide fragments using methods described below. Synthetic production of hML allows the incorporation of unnatural amino acids or synthetic functionalities such as polyethylene glycol. 15 Previously, a mutant of the serine protease subtilisin BPN, subtiligase (S221C/P225A) was engineered to efficiently ligate peptide esters in aqueous solution (Abrahmsen *et al.*, *Biochem.*, 30:4151-4159 [1991]). It has now been shown that synthetic peptides can be enzymatically ligated in a sequential manner to produce enzymatically active long peptides and proteins such as ribonuclease A (Jackson *et al.*, 20 *Science*, [1994]). This technology, described in more detail below, has enabled us to chemically synthesize long proteins that previously could be made only with recombinant DNA technology.

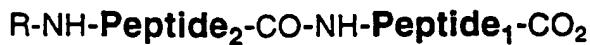
A general strategy for hML153 synthesis using subtiligase is shown (Scheme 1). Beginning with a fully deprotected peptide corresponding to the C-terminal 25 fragment of the protein, an N-terminal protected, C-terminal activated ester peptide is added along with subtiligase. When the reaction is complete, the product is isolated by reverse phase HPLC and the protecting group is removed from the N-terminus. The next peptide fragment is ligated, deprotected and the process is repeated using successive peptides until full length protein is obtained. The process is similar to 30 solid phase methodology in that an N-terminal protected C-terminal activated peptide is ligated to the N-terminus of the preceding peptide and protein is synthesized in a C->N direction. However because each coupling results in addition of up to 50 residues and the products are isolated after each ligation, much longer highly pure proteins can be synthesized in reasonable yields.

35

## Schem 1. Strategy for Synthesis of hML Using Subtiligase



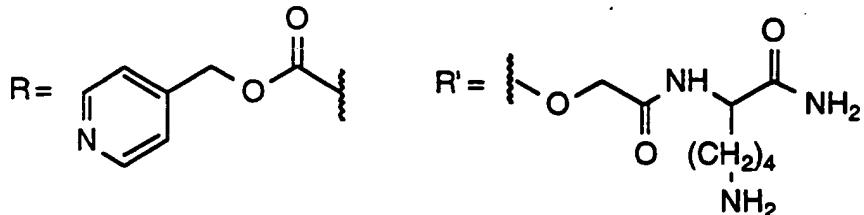
↓ 1) Subtiligase



↓ 2) Zn/CH<sub>3</sub>CO<sub>2</sub>H



↓ 3) repeat 1 + 2



5      Based on our knowledge of the sequence specificity of the subtiligase as well as the amino acid sequence of the biologically active "epo-domain" of hML, we divided hML<sub>153</sub> into seven fragments 18-25 residues in length. Test ligation tetrapeptides were synthesized to determine suitable ligation junctions for the 18-25mer's. Table 13 shows the results of these test ligations.

10

TABLE 13

**hML Test Ligations.** Donor and nucleophile peptides were dissolved at 10 mM in 100 mM tricine (pH 7.8) at 22°C. Ligase was added to a final concentration of 10 μM from a 1.6 mg/mL stock (~70 μM) and the ligation allowed to proceed overnight.

15      Yields are based on % ligation vs. hydrolysis of the donor peptides.

Site	Donor (glc-K-NH <sub>2</sub> )	Nucleophile-NH <sub>2</sub>	%Hydrolysis	%Ligation
1 (23/24)	HVLH (SEQ ID NO: 89)	SRLS (SEQ ID NO: 90)	92	08
(22/23)	SHVL (SEQ ID NO: 91)	HSRL (SEQ ID NO: 92)	48	52
2 (46/47)	AVDF (SEQ ID NO: 93)	SLGE (SEQ ID NO: 94)	22	78
3 (69/70)	AVTL (SEQ ID NO: 95)	LLEG (SEQ ID NO: 96)	53	47
4 (89/90)	LSSL (SEQ ID NO: 97)	LGQL (SEQ ID NO: 98)	95	05
(88/89)	C(acm)LSS (SEQ ID NO: 99)	LLGQ (SEQ ID NO: 100)	00	00
(90/91)	S\$LL (SEQ ID NO: 101)	GQLS (SEQ ID NO: 102)	45	55
(88/89)	CLSS (SEQ ID NO: 103)	LLGQ (SEQ ID NO: 100)	90	10
5(107/108)	LQSL (SEQ ID NO: 104)	LGTQ (SEQ ID NO: 105)	99	01
(106/107)	ALQS (SEQ ID NO: 106)	LLGT (SEQ ID NO: 107)	70	30
6(128/129)	NAIF (SEQ ID NO: 108)	LSFQ (SEQ ID NO: 109)	60	40

Based on these experiments, the ligation peptides indicated in Table 14 should be efficiently ligated by the subtiligase. A suitable protecting group for the N-terminus of each donor ester peptide was needed to prevent self-ligation. We chose an isonicotinyl (iNOC) protecting group (Veber *et al.*, *J. Org. Chem.*, 42:3286-3289 [1977]) because it is water soluble, it can be incorporated at the last step of solid phase peptide synthesis and it is stable to anhydrous HF used to deprotect and cleave peptides from the solid phase resin. In addition, it can be removed from the peptide after each ligation under mild reducing conditions (Zn/CH<sub>3</sub>CO<sub>2</sub>H) to afford a free N-terminus for subsequent ligations. A glycolate-lysyl-amide (glc-K-NH<sub>2</sub>) ester was used for C-terminal activation based on previous experiments which showed this to be efficiently acylated by subtiligase (Abrahmsen *et al.*, *Biochem.*, 30:4151-4159 [1991]). The iNOC-protected, glc-K-amide activated peptides can be synthesized

using standard solid phase methods as outlined (Scheme 2). The peptides are then sequentially ligated until the full protein is produced and the final product refolded *in vitro*. Based on homology with EPO, disulfide pairs are believed to be formed between cysteine residues 7 and 151 and between 28 and 85. Oxidation of the disulfides may be  
5 accomplished by simply stirring the reduced material under an oxygen atmosphere for several hours. The refolded material can then be purified by HPLC and fractions containing active protein pooled and lyophilized. As an alternative, disulfides can be differentially protected to control sequential oxidation between specific disulfide pairs. Protection of cysteines 7 and 151 with acetamidomethyl (acm) groups would ensure  
10 oxidation of 28 and 85. The acm groups could then be removed and residues 7 and 151 oxidized. Conversely, residues 28 and 85 could be acm protected and oxidized in case sequential oxidation is required for correct folding. Optionally, Cysteins 28 and 85 may be substituted with another natural or unnatural residue other than Cys to insure proper oxidation of cysteins 7 and 151.

15

TABLE 14.  
Peptide Fragments Used For Total Synthesis of h-ML Using Subtiligase

	<u>Fragment</u>	<u>Sequence</u>
20	1 (SEQ ID NO: 110)	
		iNOC-HN-SPAPPACDLRVLSKLLRDSHVL-glc-K-NH <sub>2</sub> (1-22)
25	2 (SEQ ID NO: 111)	
		INOC-HN-HSRLSQCPEVHPLPTPVLLPAVDF-glc-K-NH <sub>2</sub> (23-46)
30	3 (SEQ ID NO: 112)	
		INOC-HN-SLGEWKTQMEETKAQDILGAVTL-glc-K-NH <sub>2</sub> (47-69)
	4 (SEQ ID NO: 113)	
35		INOC-HN-LLEGVMAARGQLGPTCLSSLL-glc-K-NH <sub>2</sub> (70-90)

5 (SEQ ID NO: 114)

INOC-HN-GQLSGQVRLLL GALQS-glc-K-NH<sub>2</sub> (90-106)

5 6 (SEQ ID NO: 115)

INOC-HN-LLGTQLPPQGRRTAHKDPNAIF-glc-K-NH<sub>2</sub> (107-128)

7 (SEQ ID NO: 116)

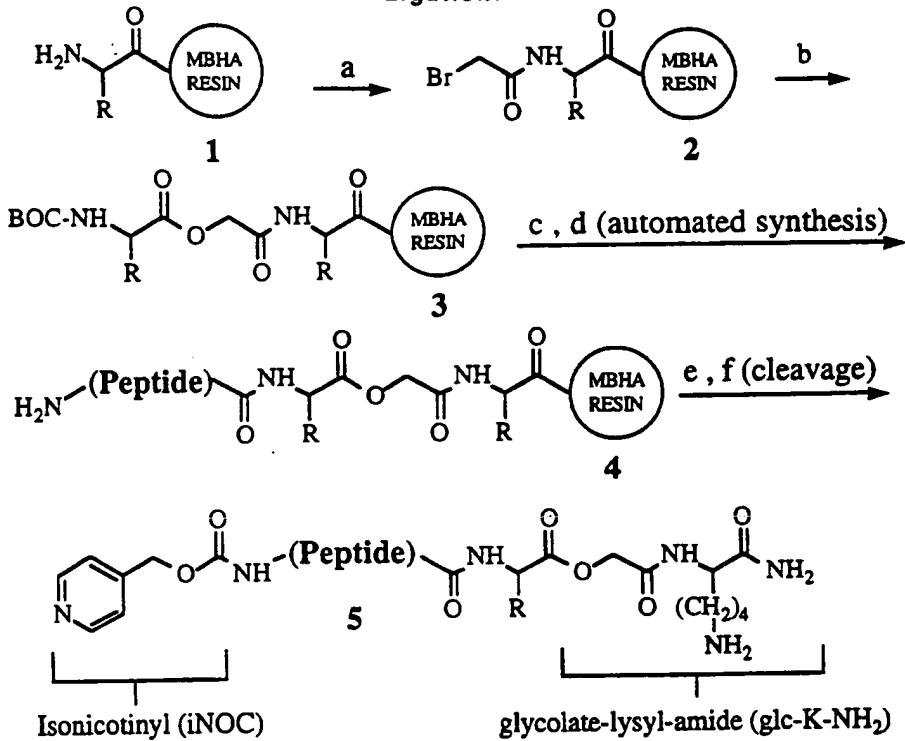
10

H<sub>2</sub>N-LSFQHLLRGKVRFLMLVGGSTLCVR-CO<sub>2</sub> (129-153)

Peptide ligations are carried out at 25°C in 100mM tricine, pH 8 (freshly prepared and degassed by vacuum filtration through a 5  $\mu$ M filter). Typically the C-terminal fragment is dissolved in buffer (2-5 mM peptide) and a 10x stock solution of subtiligase (1 mg/ml in 100mM tricine, pH 8) is added to bring the final enzyme concentration to ~ 5  $\mu$ M. A 3-5 molar excess of the glc-K-NH<sub>2</sub> activated donor peptide is then added as a solid, dissolved, and the mixture allowed to stand at 25°C. The ligations are monitored by analytical reverse phase C18 HPLC (CH<sub>3</sub>CN/H<sub>2</sub>O gradient with 0.1% TFA). The ligation products are purified by preparative HPLC and lyophilized. Isonicotinyl (iNOC) deprotection was performed by stirring HCl activated zinc dust with the protected peptide in acetic acid. The zinc dust is removed by filtration and the acetic acid evaporated under vacuum. The resulting peptide can be used directly in the next ligation and the process is repeated. Synthetic hML153 can be ligated by procedures analogous to those described above to synthetic or recombinant hML154-332 to produce synthetic or semisynthetic full length hML.

Synthetic hML has many advantages over recombinant. Unnatural side chains can be introduced in order to improve potency or specificity. Polymer functionalities such as polyethylene glycol can be incorporated to improve duration of action. For example, polyethylene glycol can be attached to lysine residues of the individual fragments (Table 14) before or after one or more ligation steps have been performed. Protease sensitive peptide bonds can be removed or altered to improve stability *in vivo*. In addition, heavy atom derivatives can be synthesized to aid in structure determination.

**Scheme 2. Solid Phase Synthesis of Peptid Fragments for Segment Ligation.**



5 a) Lysyl-paramethylbenzhydrylamine (MBHA) resin 1 (0.63 meq./gm., Advanced ChemTech) is stirred with bromoacetic acid (5 eq.) and diisopropyl carbodiimide (5 eq.) for 1 h. at 25°C in dimethylacetamide (DMA) to afford the bromoacetyl derivative 2. b) The resin is washed extensively with DMA and individual Boc-protected amino acids (3 eq., Bachem) are esterified by stirring with sodium bicarbonate (6 eq.) in dimethylformamide (DMF) for 24 h. at 50°C to afford the corresponding glycolate-phenylalanyl-amide-resin 3. The amino acetylated resin 3 is washed with DMF (3x) and dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) (3x) and can be stored at room temperature for several months. The resin 3 can then be loaded into an automated peptide synthesizer (Applied Biosystems 430A) and the peptides elongated using standard solid phase procedures

10 15 c) The N- $\alpha$ -Boc group is removed with a solution of 45% trifluoroacetic acid in CH<sub>2</sub>Cl<sub>2</sub>. d) Subsequent Boc-protected amino acids (5 eq.) are preactivated using benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP, 4 eq.) and N-methylmorpholine (NMM, 10 eq.) in DMA and coupled for 1-2 h.

20 e) The final N- $\alpha$ -Boc group is removed (TFA/CH<sub>2</sub>Cl<sub>2</sub>) to afford 4 and the isonicotinyl (iNOC) protecting group is introduced as described previously (4) via stirring with of

4-is nicotinyl-2-4-dinitr phenyl carbonate (3 eq.) and NMM (6 eq.) in DMA at 25°C for 24 h. f) Cleavage and deprotection of the peptide via treatment with anhydrous HF (5% anisole/ 5% ethylmethyl sulfide) at 0°C for 1 h. affords the iNOC-protected, glycolate-lys-amide activated peptide 5 which is purified by reverse phase 5 C18 HPLC (CH<sub>3</sub>CN/H<sub>2</sub>O gradient, 0.1% TFA). The identity of all substrates is confirmed by mass spectrometry.

\* \* \* \*

#### SUPPLEMENTAL ENABLEMENT

The invention as claimed is enabled in accordance with the above specification 10 and readily available references and starting materials. Nevertheless, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) the cell line listed below:

15 *Escherichia coli*, DH10B-pBSK-hmp/ 1.8, ATCC accession no. CRL 69575, deposited February 24, 1994.

15 Plasmid, pSVI5.ID.LL.MLORF, ATCC accession no. CRL 75958, deposited December 2, 1994; and

CHO DP-12 cells, ML 1/50 MCB (labeled #1594), ATCC accession no. CRL 11770, deposited December 6, 1994.

20 This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treat, and subject to an 25 agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

\* \* \* \*

30 While the invention has necessarily been described in conjunction with preferred embodiments and specific working examples, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein, without departing from the spirit and scope thereof. Hence, the invention can be practiced in ways other than those specifically described herein. It is therefore 35 intended that the protection granted by letters patent hereon be limited only by the appended claims and equivalents thereof.

All references cited herein are hereby expressly incorporated by reference.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Genentech, Inc.  
Eaton, Dan L.  
de Sauvage, Frederic J.

10 (ii) TITLE OF INVENTION: THROMBOPOIETIN  
15 (iii) NUMBER OF SEQUENCES: 144

(iv) CORRESPONDENCE ADDRESS:  
15 (A) ADDRESSEE: Genentech, Inc.  
(B) STREET: 460 Point San Bruno Blvd  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 94080

20 (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
25 (D) SOFTWARE: patin (Genentech)

30 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

35 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/176553  
(B) FILING DATE: 03-JAN-1994

40 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/348657  
(B) FILING DATE: 02-DEC-1994

45 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/185607  
(B) FILING DATE: 21-JAN-1994

50 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/348658  
(B) FILING DATE: 02-DEC-1994

55 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/196689  
(B) FILING DATE: 15-FEB-1994

(vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/223263  
(B) FILING DATE: 04-APR-1994

55 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/249376  
(B) FILING DATE: 25-MAY-1994

5 (viii) ATTORNEY/AGENT INFORMATION:  
 (A) NAME: Winter, Daryl B.  
 (B) REGISTRATION NUMBER: 32,637  
 (C) REFERENCE/DOCKET NUMBER: 871P5PCT

10 (ix) TELECOMMUNICATION INFORMATION:  
 (A) TELEPHONE: 415/225-1249  
 (B) TELEFAX: 415/952-9881  
 (C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 353 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Glu	Leu	Thr	Glu	Leu	Leu	Leu	Leu	Val	Val	Met	Leu	Leu	Leu	Thr	
1															15	
Ala	Arg	Leu	Thr	Leu	Ser	Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Leu		
25															30	
Arg	Val	Leu	Ser	Lys	Leu	Leu	Arg	Asp	Ser	His	Val	Leu	His	Ser		
35															45	
Arg	Leu	Ser	Gln	Cys	Pro	Glu	Val	His	Pro	Leu	Pro	Thr	Pro	Val		
50															60	
Leu	Leu	Pro	Ala	Val	Asp	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln		
65															75	
Met	Glu	Glu	Thr	Lys	Ala	Gln	Asp	Ile	Leu	Gly	Ala	Val	Thr	Leu		
80															90	
Leu	Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln	Leu	Gly	Pro	Thr		
95															105	
Cys	Leu	Ser	Ser	Leu	Leu	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu		
110															120	
Leu	Leu	Gly	Ala	Leu	Gln	Ser	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Pro		
125															135	
Gln	Gly	Arg	Thr	Thr	Ala	His	Lys	Asp	Pro	Asn	Ala	Ile	Phe	Leu		
140															150	
Ser	Phe	Gln	His	Leu	Leu	Arg	Gly	Lys	Val	Arg	Phe	Leu	Met	Leu		
155															165	
Val	Gly	Gly	Ser	Thr	Leu	Cys	Val	Arg	Arg	Ala	Pro	Pro	Thr	Thr		
170															180	
Ala	Val	Pro	Ser	Arg	Thr	Ser	Leu	Val	Leu	Thr	Leu	Asn	Glu	Leu		
185															195	
60	Pro	Asn	Arg	Thr	Ser	Gly	Leu	Leu	Glu	Thr	Asn	Phe	Thr	Ala	Ser	
200															210	

	Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly Phe		
	215	220	225
5	Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu		
	230	235	240
	Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn		
10	245	250	255
	Gly Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly		
	260	265	270
15	Ala Pro Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro		
	275	280	285
	Pro Asn Leu Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro		
	290	295	300
20	Thr Gly Gln Tyr Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro Thr		
	305	310	315
	Pro Val Val Gln Leu His Pro Leu Leu Pro Asp Pro Ser Ala Pro		
25	320	325	330
	Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn Thr Ser Tyr Thr His		
	335	340	345
30	Ser Gln Asn Leu Ser Gln Glu Gly		
	350	353	

## (2) INFORMATION FOR SEQ ID NO:2:

35	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1795 bases	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
40	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
	TCTTCCTACC CATCTGCTCC CCAGAGGGCT GCCTGCTGTG CACTTGGGTC 50	
45	CTGGAGCCCT TCTCCACCCG GATAGATTCC TCACCCCTTGG CCCGCCTTG 100	
	CCCCACCCCTA CTCTGCCAG AAGTGCAAGA GCCTAAGCCG CCTCCATGGC 150	
50	CCCAGGAAGG ATTCAGGGGA GAGGCCCAA ACAGGGAGCC ACGCCAGCCA 200	
	GACACCCCGG CCAGAATGGA GCTGACTGAA TTGCTCCTCG TGGTCATGCT 250	
55	TCTCCTAACT GCAAGGCTAA CGCTGTCCAG CCCGGCTCCT CCTGCTTGTG 300	
60	ACCTCCGAGT CCTCAGTAAA CTGCTTCGTG ACTCCCATGT CCTTCACAGC 350	

AGACTGAGCC AGTGCCAGA GGTCACCCCT TTGCCTACAC CTGTCCTGCT 400  
5 GCCTGCTGTG GACTTTAGCT TGGGAGAATG GAAAACCCAG ATGGAGGAGA 450  
CCAAGGCACA GGACATTCG GGAGCAGTGA CCCTTCTGCT GGAGGGAGTG 500  
10 ATGGCAGCAC GGGGACAACG GGGACCCACT TGCCCTCTCAT CCCTCCTGGG 550  
GCAGCTTTCT GGACAGGTCC GTCTCCTCCT TGGGGCCCTG CAGAGCCTCC 600  
15 TTGGAACCCA GCTTCCTCCA CAGGGCAGGA CCACAGCTCA CAAGGATCCC 650  
AATGCCATCT TCCTGAGCTT CCAACACCTG CTCCGAGGAA AGGTGCGTTT 700  
CCTGATGCTT GTAGGAGGGT CCACCCCTCTG CGTCAGGCGG GCCCCACCCA 750  
25 CCACAGCTGT CCCCCAGCAGA ACCTCTCTAG TCCTCACACT GAACGAGCTC 800  
CCAAACAGGA CTTCTGGATT GTTGGAGACA AACTTCACTG CCTCAGCCAG 850  
30 AACTACTGGC TCTGGGCTTC TGAAGTGGCA GCAGGGATTC AGAGCCAAGA 900  
TTCCCTGGTCT GCTGAACCAA ACCTCCAGGT CCCTGGACCA AATCCCCGGA 950  
TACCTGAACA GGATACACGA ACTCTGAAT GGAACTCGTG GACTCTTCC 1000  
40 TGGACCCCTCA CGCAGGACCC TAGGAGCCCC GGACATTTCC TCAGGAACAT 1050  
CAGACACAGG CTCCCTGCCA CCCAACCTCC AGCCTGGATA TTCTCCTTCC 1100  
45 CCAACCCATC CTCCTACTGG ACAGTATACTG CTCTTCCCTC TTCCACCCAC 1150  
CTTGCCCCACC CCTGTGGTCC AGCTCCACCC CCTGCTTCCT GACCCTCTG 1200  
CTCCAACGCC CACCCCTACC AGCCCTCTTC TAAACACATC CTACACCCAC 1250  
55 TCCCAGAACATC TGTCTCAGGA AGGGTAAGGT TCTCAGACAC TGCCGACATC 1300  
AGCATTGTCT CATGTACAGC TCCCTCCCT GCAGGGCGCC CCTGGGAGAC 1350  
60

AACTGGACAA GATTCCTAC TTTCTCCTGA AACCCAAAGC CCTGGTAAAA 1400  
 5 GGGATACACA GGACTGAAA GGGAAATCATT TTTCACTGTA CATTATAAAC 1450  
 CTTCAGAAC C TATTTTTTA AGCTATCAGC AATACTCATC AGAGCAGCTA 1500  
 10 GCTCTTTGGT CTATTTCTG CAGAAATTG CAACTCACTG ATTCTCTACA 1550  
 TGCTCTTTT CTGTGATAAC TCTGCAAAGG CCTGGGCTGG CCTGGCAGTT 1600  
 15 GAACAGAGGG AGAGACTAAC CTTGAGTCAG AAAACAGAGA AAGGGTAATT 1650  
 TCCTTGCTT CAAATTCAAG GCCTTCCAAC GCCCCCACCC CCTTTACTAT 1700  
 20 CATTCTCAGT GGGACTCTGA TCCCATATT C TAAACAGATC TTTACTCTTG 1750  
 25 AGAAATGAAT AAGCTTTCTC TCAGAAAAAA AAAAAAAA AAAAAA 1795

30 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 42 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu	Leu	Leu	Val	Val	Met	Leu	Leu	Leu	Thr	Ala	Arg	Leu	Thr	Leu
1					5				10			15		
Ser	Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Leu	Arg	Val	Leu	Ser	Lys
40					20			25			30			
Leu	Leu	Arg	Asp	Ser	His	Val	Leu	His	Ser	Arg	Leu			
45				35				40			42			

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 390 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAATTCCCTGG AATACCAGCT GACAATGATT TCCTCCTCAT CTTTCAACCT 50  
 60 CACCTCTCCT CATCTAAGAA TTGCTCCTCG TGGTCATGCT TCTCCTAACT 100

5           GCAAGGCTAA CGCTGTCCAG CCCGGCTCCT CCTGCTTGTG ACCTCCGAGT 150  
CCTCAGTAAA CTGCTTCGTG ACTCCCATGT CCTTCACAGC AGACTGGTGA 200  
10           GAACTCCCAA CATTATCCCC TTTATCCGCG TAACTGGTAA GACACCCATA 250  
CTCCCAGGAA GACACCATCA CTTCCCTCTAA CTCCCTTGACC CAATGACTAT 300  
15           TCTTCCCATA TTGTCCCCAC CTACTGATCA CACTCTCTGA CAAGAATTAT 350  
TCTTCACAAT ACAGCCCCGA TTTAAAAGCT CTCGTCTAGA 390  
20

(2) INFORMATION FOR SEQ ID NO:5:  
25           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 390 bases  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear  
30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
CTTAAGGACC TTATGGTCGA CTGTTACTAA AGGAGGGAGTA GAAAGTTGGA 50  
35           GTGGAGAGGA GTAGATTCTT AACGAGGGAGC ACCAGTACGA AGAGGATTGA 100  
CGTTCCGATT GCGACAGGTC GGGCCGAGGA GGACGAACAC TGGAGGCTCA 150  
40           GGAGTCATTT GACGAAGCAC TGAGGGTACA GGAAGTGTG TCTGACCACT 200  
45           CTTGAGGGTT GTAATAGGGG AAATAGGCGC ATTGACCATT CTGTGGGTAT 250  
GAGGGTCCTT CTGTGGTAGT GAAGGAGATT GAGGAACCTGG GTTACTGATA 300  
50           AGAAGGGTAT AACAGGGGTG GATGACTAGT GTGAGAGACT GTTCTTAATA 350  
AGAAGTGTAA TGTCGGCGT AAATTTCTGA GAGCAGATCT 390  
55

(2) INFORMATION FOR SEQ ID NO:6:  
60           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 332 amino acids

(B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

5	Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu	1	5	10	15
10	Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro	20	25	30	:
15	Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp	35	40	45	
20	Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala	50	55	60	
25	Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met	65	70	75	
30	Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu	80	85	90	
35	Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln	95	100	105	
40	Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala	110	115	120	
45	His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu	125	130	135	
50	Arg Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu	140	145	150	
55	Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr	155	160	165	
60	Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn Arg Thr Ser Gly	170	175	180	
65	Leu Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg Thr Thr Gly Ser	185	190	195	
70	Gly Leu Leu Lys Trp Gln Gln Gly Phe Arg Ala Lys Ile Pro Gly	200	205	210	
75	Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly Tyr	215	220	225	
80	Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe	230	235	240	
85	Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser	245	250	255	
90	Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro Gly	260	265	270	
95	Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu	275	280	285	

	Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu His		
	290	295	300
5	Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser		
	305	310	315
	Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln		
	320	325	330
10	Glu Gly		
	332		

15 (2) INFORMATION FOR SEQ ID NO:7:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 166 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

	Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr			
	1	5	10	15
25	Leu Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala			
	20	25	30	
30	Glu His Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys			
	35	40	45	
	Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala			
	50	55	60	
35	Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu			
	65	70	75	
	Arg Gly Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro			
40	80	85	90	
	Leu Gln Leu His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu			
	95	100	105	
45	Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser			
	110	115	120	
	Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala			
	125	130	135	
50	Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg			
	140	145	150	
	Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp			
55	155	160	165	
	Arg			
	166			

## (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 328 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

10	Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu	1	5	10	15
	Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro				
15	20	25	30		
	Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp				
	35	40	45		
20	Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala	50	55	60	
	Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met	65	70	75	
25	Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu	80	85	90	
	Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln	95	100	105	
30	Ser Leu Leu Gly Thr Gln Gly Arg Thr Thr Ala His Lys Asp Pro	110	115	120	
	Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val	125	130	135	
35	Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg	140	145	150	
40	Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu	155	160	165	
	Thr Leu Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr	170	175	180	
45	Asn Phe Thr Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys	185	190	195	
50	Trp Gln Gln Gly Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln	200	205	210	
	Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile	215	220	225	
55	His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe Pro Gly Pro Ser	230	235	240	
	Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser Gly Thr Ser Asp	245	250	255	
60					

	Thr	Gly	Ser	Leu	Pro	Pro	Asn	Leu	Gln	Pro	Gly	Tyr	Ser	Pro	Ser
				260				265						270	
5	Pro	Thr	His	Pro	Pro	Thr	Gly	Gln	Tyr	Thr	Leu	Phe	Pro	Leu	Pro
				275				280					285		
	Pro	Thr	Leu	Pro	Thr	Pro	Val	Val	Gln	Leu	His	Pro	Leu	Leu	Pro
				290				295					300		
10	Asp	Pro	Ser	Ala	Pro	Thr	Pro	Thr	Ser	Pro	Leu	Leu	Asn		
				305				310					315		
	Thr	Ser	Tyr	Thr	His	Ser	Gln	Asn	Leu	Ser	Gln	Glu	Gly		
15					320				325			328			
	(2) INFORMATION FOR SEQ ID NO:9:														
	(i) SEQUENCE CHARACTERISTICS:														
20	(A) LENGTH: 265 amino acids														
	(B) TYPE: amino acid														
	(D) TOPOLOGY: linear														
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:														
25	Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Leu	Arg	Val	Leu	Ser	Lys	Leu
	1				5				10				15		
	Leu	Arg	Asp	Ser	His	Val	Leu	His	Ser	Arg	Leu	Ser	Gln	Cys	Pro
30					20				25				30		
	Glu	Val	His	Pro	Leu	Pro	Thr	Pro	Val	Leu	Leu	Pro	Ala	Val	Asp
				35				40				45			
35	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln	Met	Glu	Glu	Thr	Lys	Ala
				50				55				60			
	Gln	Asp	Ile	Leu	Gly	Ala	Val	Thr	Leu	Leu	Leu	Gly	Val	Met	
				65				70				75			
40	Ala	Ala	Arg	Gly	Gln	Leu	Gly	Pro	Thr	Cys	Leu	Ser	Ser	Leu	Leu
				80				85				90			
	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	Gly	Ala	Leu	Gln
45					95				100				105		
	Ser	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Pro	Gln	Gly	Arg	Thr	Thr	Ala
				110				115				120			
50	His	Lys	Asp	Pro	Asn	Ala	Ile	Phe	Leu	Ser	Phe	Gln	His	Leu	Leu
				125				130				135			
	Arg	Gly	Lys	Asp	Phe	Trp	Ile	Val	Gly	Asp	Lys	Leu	His	Cys	Leu
				140				145				150			
55	Ser	Gln	Asn	Tyr	Trp	Leu	Trp	Ala	Ser	Glu	Val	Ala	Ala	Gly	Ile
				155				160				165			
	Gln	Ser	Gln	Asp	Ser	Trp	Ser	Ala	Glu	Pro	Asn	Leu	Gln	Val	Pro
60				170				175				180			

	Gly Pro Asn Pro Arg Ile Pro Glu Gln Asp Thr Arg Thr Leu Glu		
	185	190	195
5	Trp Asn Ser Trp Thr Leu Ser Trp Thr Leu Thr Gln Asp Pro Arg		
	200	205	210
	Ser Pro Gly His Phe Leu Arg Asn Ile Arg His Arg Leu Pro Ala		
	215	220	225
10	Thr Gln Pro Pro Ala Trp Ile Phe Ser Phe Pro Asn Pro Ser Ser		
	230	235	240
	Tyr Trp Thr Val Tyr Ala Leu Pro Ser Ser Thr His Leu Ala His		
	245	250	255
15	Pro Cys Gly Pro Ala Pro Pro Pro Ala Ser		
	260	265	

(2) INFORMATION FOR SEQ ID NO:10:

20	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 261 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
25	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:			
	Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu			
	1	5	10	15
30	Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro			
	20	25	30	
35	Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp			
	35	40	45	
	Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala			
	50	55	60	
40	Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met			
	65	70	75	
	Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu			
	80	85	90	
45	Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln			
	95	100	105	
50	Ser Leu Leu Gly Thr Gln Gly Arg Thr Thr Ala His Lys Asp Pro			
	110	115	120	
	Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Asp			
	125	130	135	
55	Phe Trp Ile Val Gly Asp Lys Leu His Cys Leu Ser Gln Asn Tyr			
	140	145	150	
	Trp Leu Trp Ala Ser Glu Val Ala Ala Gly Ile Gln Ser Gln Asp			
	155	160	165	
60				

	Ser Trp Ser Ala Glu Pro Asn Leu Gln Val Pro Gly Pro Asn Pro		
	170	175	180
5	Arg Ile Pro Glu Gln Asp Thr Arg Thr Leu Glu Trp Asn Ser Trp		
	185	190	195
	Thr Leu Ser Trp Thr Leu Thr Gln Asp Pro Arg Ser Pro Gly His		
	200	205	210
10	Phe Leu Arg Asn Ile Arg His Arg Leu Pro Ala Thr Gln Pro Pro		
	215	220	225
15	Ala Trp Ile Phe Ser Phe Pro Asn Pro Ser Ser Tyr Trp Thr Val		
	230	235	240
	Tyr Ala Leu Pro Ser Ser Thr His Leu Ala His Pro Cys Gly Pro		
	245	250	255
20	Ala Pro Pro Pro Ala Ser		
	260 261		

## (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

25	(A) LENGTH: 7849 bases
	(B) TYPE: nucleic acid
	(C) STRANDEDNESS: single
	(D) TOPOLOGY: linear

## 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

35	CCAGCCTCC TTTCTCTTGT TCCCTGGTCA TGCCTGCCTC CCTGTCTCCT 50
	GCTCTCTCCCT CCCACACACA CCCACTATCC TCCCAAGCTAT CCCTACACCC 100
40	TCCTTCCTAA TCTTGGGAGA CATCTCGTCT GGCTGGACGG GAAAATTCCA 150
	GGATCTAGGC CACACTCTC AGCAGACATG CCCATCCTTG GGGAGGAGGA 200
45	ACAGGAGAGA GCCTGAGGAA GTTCTGGGG ACAGGGGGAT GATGGGATCA 250
	AGGTCAGGCC AGGAAGCCCC TGAGGACAGA GACTGTGGGG AGACTGGAC 300
50	TGGGAAGAAA GCAAAGGAGC TAGAGCCAGG GCCAAAGGAA AAGGGGGGCC 350
	AGCAGGGAGG TATTTGGGG GGAGGTCCAG CAGCTGTCTT TCCTAAGACA 400
55	GGGACACATG GGCCTGGTTA TTCCTCTTGT CACATGTGGA ACGGTAGGAG 450
60	ATGGAAGACG GAGACAGAAC AAGCAAAGGA GGGCCCTGGG CACAGAGGTC 500

TGTGTGTGTA GCCATCCAAG CCACTGGACC CCAGCAGACG AGCACCTAAG 550  
5 CTCAGGCTTA ACCCAGTGCA CGTGTGCGCA CATAATGTG CCCCCCACCT 600  
GACAGTCCAC TCAACCCGTC CAAACCCTTT CCCCCATAACA CCAACCCATA 650  
10 ACAGGAGATT TCTCTCATGT GGGCAATATC CGTGTCCCA CTTCGAAAGG 700  
GGGAATGACA AGATAGGACT CCCTAGGGGA TTACAGAAAG AAAAGCAGGA 750  
15 AAGCAAGCAT CCTGTTGGAT TTCAGCAGCA GGTATGATGT CCAGGGAAAA 800  
20 GAAATTGGA TAGCCAGGGA GTGAAAACCC CACCAATCTT AAACAAGACC 850  
TCTGTGCTTC TTCCCCAGCA ACACAAATGT CCTGCCAGAT TCCTCCTGGA 900  
25 AAAAAACTTC TGCTCCTGTC CCCCTCCAGG TCCCAGGTTG CCCATGTCCA 950  
GGAAAAGATG GATCCCCCTA TCCAAATCTT CTCCGTGGTG TGTGTGGGTG 1000  
30 GAGGAGTGGGA CCCTGGTCCA GGCAGGGCT CCAGGGAAAGA GAAGGCGTCA 1050  
35 CTTCCGGGGG CCTTCACCAG TGTCTGGTGG CTCCCTCTC TGATTGGGCA 1100  
GAAGTGGCCC AGGCAGGCGT ATGACCTGCT GCTGTGGAGG GGCTGTGCC 1150  
40 CACCGCCACA TGTCTTCTA CCCATCTGCT CCCCCAGAGGG CTGCCTGCTG 1200  
TGCACTTGGG TCCTGGAGCC CTTCTCCACC CGGTGAGTGG CCAGCAGGGT 1250  
45 GTGGGGTTAT GTGAGGGTAG AAAGGACAGC AAAGAGAAAT GGGCTCCAG 1300  
50 CTGGGGGAGG GGCAGGCAAA CTGGAACCTA CAGGCAGTGA CCTTTGTGCA 1350  
GAAGAGTGTGTA GCCTTCCCAG AATGGGAGGA GCAGGGCAGA GCAGGGTAG 1400  
55 GGGGTGGGGT GCTGGTTCT GAGGGACTGA TCACCTACTT GGTGGAATAC 1450  
60 AGCACAGCCC TGGCTGGCCC TAAGGAAAGG GGACATGAGC CCAGGGAGAA 1500

AATAAGAGAG GGAGCTGCAC TTAGGGCTTA GCAAACACAG TAGTAAGATG 1550  
5 GACACAGCCC CAATCCCCAT TCTTAGCTGG TCATTCCTCG TTAGCTTAAG 1600  
GTTCTGAATC TGGTGCTGGG GAAGCTGGC CAGGCAAGCC AGGGCGCAAG 1650  
10 GAGAGGGTAA TGGGAGGAGG GCCCACTCAT GTTGACAGAC CTACAGGAAA 1700  
TCCCAATATT GAATCAGGTG CAAGCCTCTT TGCACAACTT GTGAAAGGAG 1750  
15 GAGGAAGCCA TGTGGGGGT CCTGTGAAGG AACCGGAAGG GGTTCTGCCA 1800  
20 AGGGGGCAGG GAGGCAGGTG TGAGCTATGA GACAGATATG TTAGTGGCG 1850  
CCTAAGACAA GGTAAGCCCC TAAGGTGGGC ATCACCCAGC AGGTGCCCGT 1900  
25 TCCTGGGCAG CTGGTCTCAG GAAGGAAGTC CCAGAACTGT TAGCCCATCT 1950  
CTTGGCCTCA GATAATGGAG TATTCAGGA CTTGGAGTCC AAAGAAAAGC 2000  
30 TCCAGTGGCT TTATGTGTGG GGGTAGATAG GGAAAGAATA GAGGTTAATT 2050  
TCTCCCATAAC CGCCTTTAA TCCTGACCTC TAGTGGTCCC AGTTACAGCT 2100  
35 TTGTGCAGTT CCCCTCCCCA GCCCCACTCC CCACCGCAGA AGTTACCCCT 2150  
40 CAACATATTG CGCCCGTTTG CCAGTTCCTC ACCCAGGCC TGCATCCCAT 2200  
TTTCCACTCT CTTCTCCAGG CTGAAGCCAC AATACTTTCC TTCTCTATCC 2250  
45 CCATCCCAGA TTTTCTCTGA CCTAACAAACC AAGGTTGCTC AGAATTAAAG 2300  
GCTAATTAAG ATATGTGTGT ATACATATCA TGTCCTGCTG CTCTCAGCAG 2350  
50 GGGTAGGTGG CACCAAATCC GTGTCCGATT CACTGAGGAG TCCTGACAAA 2400  
55 AAGGAGACAC CATATGCTTT CTTGCTTTCT TTCTTTCTTT CTTCTTTTT 2450  
TTTTTTTTGA GACGGAGTTT CACTCTTATT GCCCAGGCTG GAGTGCAATG 2500  
60 GTGCGATCTC GGCTCACCAAC AAACCTCCGC CTCCCAGGTA CAAGCGATTG 2550

150 TCCGTGCTCA GCCTCCCAAG TAGCTTGGAT TACAGGCATG AGCCACCACA 2600  
5 CCCTGCTAGT TTTTTTGTAT TTCGTAGAGC CGGGGTTTCA CCATGTTAGT 2650  
10 GAGGCTGGTG GCGAACTCCT GACCTCAGGT GATCCACCCG CCTTGGACTC 2700  
15 CAAAGTGCT GGGATTACAG GCATGAGCCA CTGCACCCGG CACACCATAT 2750  
20 GCTTTCATCA CAAGAAAATG TGAGAGAATT CAGGGCTTTG GCAGTTCCAG 2800  
GCTGGTCAGC ATCTCAAGCC CTCCCCAGCA TCTGTTCAACC CTGCCAGGCA 2850  
25 GTCTCTTCCT AGAAACTTGG TTAAATGTTG ACTCTTCTTG CTACTTTCAAG 2900  
GATAGATTCC TCACCCCTTGG CCCGCCCTTG CCCCACCCCTA CTCTGCCAG 2950  
30 AAGTGCAAGA GCCTAAGCCG CCTCCATGGC CCCAGGAAGG ATTCAAGGGGA 3000  
35 GAGGCCCAA ACAGGGAGCC ACGCCAGCCA GACACCCGG CCAGAATGGGA 3050  
GCTGACTGGT GAGAACACAC CTGAGGGGCT AGGGCCATAT GGAAACATGA 3100  
40 CAGAAGGGGA GAGAGAAAGG AGACACGCTG CAGGGGCAG GAAGCTGGGG 3150  
GAACCCATTG TCCCCAAAAT AAGGGGTCTG AGGGGTGGAT TCCCTGGGTT 3200  
45 TCAGGTCTGG GTCCTGAATG GGAATTCTTG GAATACCAGC TGACAATGAT 3250  
50 TTCCCTCCTCA TCTTTCAACC TCACCTCTCC TCATCTAAGA ATTGCTCCTC 3300  
GTGGTCATGC TTCTCCTAAC TGCAAGGCTA ACGCTGTCCA GCCCGGCTCC 3350  
55 TCCCTCACAG CAGACTGGTG AGAACTCCCA ACATTATCCC CTTTATCCGC 3400  
GTAACTGGTA AGACACCCAT ACTCCCAGGA AGACACCATC ACTTCCTCTA 3450  
60 ACTCCTTGAC CCAATGACTA TTCTTCCCAT ATTGTCCCCA CCTACTGATC 3500  
ACTCCTTGAC CCAATGACTA TTCTTCCCAT ATTGTCCCCA CCTACTGATC 3550

ACACTCTCTG ACAAGAATTA TTCTTCACAA TACAGCCCGC ATTTAAAAGC 3600  
5 TCTCGTCTAG AGATAGTACT CATGGAGGAC TAGCCTGCTT ATTAGGCTAC 3650  
CATAGCTCTC TCTATTTCAAG CTCCCTTCTC CCCCCACCAA TCTTTTTCAA 3700  
10 CAGAGCCAGT GCCCAGAGGT TCACCCTTG CCTACACCTG TCCTGCTGCC 3750  
TGCTGTGGAC TTTAGCTTGG GAGAATGGAA AACCCAGATG GTAAGAAAGC 3800  
15 CATCCCTAAC CTTGGCTTCC CTAAGTCCTG TCTTCAGTTT CCCACTGCTT 3850  
20 CCCATGGATT CTCCAACATT CTTGAGCTTT TTAAAAATAT CTCACCTTCA 3900  
GCTTGGCCAC CCTAACCCAA TCTACATTCA CCTATGATGA TAGCCTGTGG 3950  
25 ATAAGATGAT GGCTTGCAGG TCCAATATGT GAATAGATTT GAAGCTGAAC 4000  
ACCATGAAAA GCTGGAGAGA AATCGCTCAT GGCCATGCCT TTGACCTATT 4050  
30 CCYGTTCAGT CTTCTTAAAT TGGCATGAAG AAGCAAGACT CATATGTCAT 4100  
35 CCACAGATGA CACAAAGCTG GGAAGTACCA CTAAAATAAC AAAAGACTGA 4150  
ATCAAGATTC AAATCACTGA AAGACTAGGT CAAAAACAAG GTGAAACAAC 4200  
40 AGAGATATAA ACTTCTACAT GTGGGCCGGG GGCTCACGCC TGTAATCCCA 4250  
GCACTTTGGG AGGCCGAGGC AGGCAGATCA CCTGAGGGCA GGAGTTTGAG 4300  
45 AGCAGCCTGG CCAACATGGC GAAACCCGT CTCTACTAAG AATACAAAAT 4350  
50 TAGCCGGCA TGGTAGTGCA TGCCTGTAAT CCCAGCTACT TGGAAGGCTG 4400  
AAGCAGGAGA ATCCCTTGAA CCCAGGAGGT GGAGGTTGTA GTGAGCTGAG 4450  
55 ATCATGCCAA TGCACCTCCAG CCTGGGTGAC AAGAGCAAAA CTCCGTCTCA 4500  
60 AAAAGAAAAA AAAATTCTAC ATGTGTAAT TAATGAGTAA AGTCCTATTG 4550

CAGCTTCAG GCCACAATGC CCTGCCTCCA TCATTTAACGC CTCTGGCCCT 4600  
5 AGCACTTCCT ACGAAAAGGA TCTGAGAGAA TAAATTGCC CCCAAACTTA 4650  
CCATGTAACA TTACTGAAGC TGCTATTCTT AAAGCTAGTA ATTCTTGTCT 4700  
10 GTTGATGTT TAGCATCCCC ATTGTGGAAA TGCTCGTACA GAACTCTATT 4750  
CCGAGTGGAC TACACTAAA TATACTGGCC TGAAACACCGG ACATCCCCCT 4800  
15 GAAGACATAT GCTAATTAT TAAGAGGGAC CATATTAAAC TAACATGTGT 4850  
CTAGAAAGCA GCAGCCTGAA CAGAAAGAGA CTAGAAGCAT GTTTATGGG 4900  
20 CAATAGTTA AAAAACTAAA ATCTATCCTC AAGAACCTA GCGTCCCTTC 4950  
TTCCTTCAGG ACTGAGTCAG GGAAGAAGGG CAGTTCTAT GGGTCCCTTC 5000  
TAGTCCTTTC TTTTCATCCT TATGATCATT ATGGTAGAGT CTCATACCTA 5050  
30 CATTAGTTT ATTTATTATT ATTATTGAG ACGGAGTCTC ACTCTATCCC 5100  
CCAGGCTGGA GTGCAGTGGC ATGATCTCAA CTCACTGCAA CCTCAGCCTC 5150  
35 CGGATTCAA GCGATTCTCC TGCCTCAGTC TCCCAAGTAG CTGGGATTAC 5200  
40 AGGTGCCAC CACCATGCC AGCTAATTG TGTTTTTG GTAGAGATGG 5250  
GGTTTCACCA TGTTGCCAG GCTGATCTTG AACTCCTGAC CTCAGGTGAT 5300  
45 CCACCTGCCT CAGCCTCCA AAGTGTGGG ATTACAGGCG TGAGCCACTG 5350  
CACCCAGCCT TCATTCAGTT TAAAATCAA ATGATCCTAA GGTTTTGCAG 5400  
50 CAGAAAGAGT AAATTTGCAG CACTAGAACC AAGAGGTAAA AGCTGTAACA 5450  
GGGCAGATT CAGCAACCTA AGAAAAAAGG AGCTCTTCTC ACTGAAACCA 5500  
AGTGTAAGAC CAGGCTGGAC TAGAGGACAC GGGAGTTTT GAAGCAGAGG 5550  
60 CTGATGACCA GCTGTCGGGA GACTGTGAAG GAATTCCCTGC CCTGGGTGGG 5600

ACCTTGGTCC TGTCCAGTTC TCAGCCTGTA TGATTCACTC TGCTGGCTAC 5650  
5 TCCTAAGGCT CCCCCACCCGC TTTTAGTGTG CCCCTTGAGG CAGTGCCTT 5700  
10 CTCTCTTCCA TCTCTTTCTC AGGAGGAGAC CAAGGCACAG GACATTCTGG 5750  
15 GAGCAGTGAC CCTCTCTGCTG GAGGGAGTGA TGGCAGGCACG GGGACAACTG 5800  
20 GGACCCACTT GCCTCTCATC CCTCCTGGGG CAGCTTTCTG GACAGGTCCG 5850  
TCTCCTCCTT GGGGCCCTGC AGAGCCTCCT TGGAACCCAG GTAAGTCCCC 5900  
25 AGTCAAGGGA TCTGTAGAAA CTGTTCTTTT CTGACTCAGT CCCACTAGAA 5950  
GACCTGAGGG AAGAAGGGCT CTTCCAGGG A GCTCAAGGGC AGAAGAGCTG 6000  
30 ATCTACTAAG AGTGCTCCCT GCCAGCCACA ATGCCTGGGT ACTGGCATCC 6050  
TGTCTTCCT ACTTAGACAA GGGAGGCCTG AGATCTGCC CTGGTGTTC 6100  
GCCTCAGGAC CATCCTCTGC CCTCAGCTTC CTCCACAGGG CAGGACCACA 6150  
35 GCTCACAAAGG ATCCCAATGC CATCTTCCTG AGTTTCCAAC ACCTGCTCCG 6200  
AGGAAAGGTG CGTTTCCTGA TGCTTGTAGG AGGGTCCACC CTCTGCGTCA 6250  
40 GGCGGGCCCC ACCCACCACA GCTGTCCCCA GCAGAACCTC TCTAGTCCTC 6300  
45 ACACTGAACG AGCTCCAAA CAGGACTTCT GGATTGTTGG AGACAAACTT 6350  
CACTGCCTCA GCCAGAACTA CTGGCTCTGG GCTTCTGAAG TGGCAGCAGG 6400  
50 GATTCAAGAGC CAAGATTCCCT GGTCTGCTGA ACCAAACCTC CAGGTCCCTG 6450  
GACCAAATCC CCGGATACCT GAACAGGATA CACGAACCTCT TGAATGGAAC 6500  
55 TCGTGGACTC TTTCTGGAC CCTCACGCAG GACCCTAGGA GCCCCGGACA 6550  
60 TTTCTCAGG AACATCAGAC ACAGGCTCCC TGCCACCCAA CCTCCAGCCT 6600

GGATATTCTC CTTCCCCAAC CCATCCTCCT ACTGGACAGT ATACGCTCTT 6650  
5 CCCTCTTCCA CCCACCTTGC CCACCCCTGT GGTCCAGCTC CACCCCTGC 6700  
TTCTGACCC TTCTGCTCCA ACGCCCACCC CTACCAGCCC TCTTCTAAAC 6750  
10 ACATCCTACA CCCACTCCCA GAATCTGTCT CAGGAAGGGT AAGGTTCTCA 6800  
GACACTGCCG ACATCAGCAT TGTCTCATGT ACAGCTCCCT TCCCTGCAGG 6850  
15 CGCCCCCTGG GAGACAAC TG GACAAGATTT CCTACTTTCT CCTGAAACCC 6900  
20 AAAGCCCTGG TAAAAGGGAT ACACAGGACT GAAAAGGGAA TCATTTTCA 6950  
CTGTACATTA TAAACCTTCA GAAGCTATTT TTTAAGCTA TCAGCAATAC 7000  
25 TCATCAGAGC AGCTAGCTCT TTGGTCTATT TTCTGCAGAA ATTTGCAACT 7050  
CACTGATTCT CTACATGCTC TTTTCTGTG ATAATCTGC AAAGGCCTGG 7100  
30 CCTGGCCTGG CAGTTGAACA GAGGGAGAGA CTAACCTTGA GTCAGAAAAC 7150  
35 AGAGAAAGGG TAATTCCTT TGCTTCAAAT TCAAGGCCTT CCAACGCC 7200  
CATCCCCTTT ACTATCATTC TCAGTGGAC TCTGATCCC TATTCTTAAC 7250  
40 AGATCTTAC TCTTGAGAAA TGAATAAGCT TTCTCTCAGA AATGCTGTCC 7300  
CTATACACTA GACAAAAC TG AGCCTGTATA AGGAATAAT GGGAGCGCCG 7350  
45 AAAAGCTCCC TAAAAAGCAA GGGAAAGATG TTCTTCGAGG GTGGCAATAG 7400  
50 ATCCCCCTCA CCCTGCCACC CCAAAACAAA AAGCTAACAG GAAGCCTTGG 7450  
AGAGCCTCAC ACCCCAGGTA AGGCTGTGTA GACAGTTCAG TAAAGACAGG 7500  
55 ACCTGGATGT GACAGCTGAG CAAACAGCTA GAGCTTGGC AGCTCAGCAG 7550  
60 GAGGCTTGC CAGGCATGGA CGCCTGCCTC CCTCCTGTGG AGGTCAGGAG 7600

GAAGTGCAGG AAGTGGCATG AGTCAGGCTC CTTGAGCTCA CACAGCAGGA 7650

5 GAACAACTAC AAGTCAAGTA CAAGTTGAAG GCTCATTCC CAGTTCCCGC 7700

AAATGCATCT AAAAAGCAGC TCTGTGTGAC CACCATAAAC TCTGCTAGGG 7750

10 GATCTCTAAA AAGGAGTCAG GCTTATGGGG CTTTCAAAT AAGTGCTGCC 7800

TTGGTGCCTCA GGAAAAGGTT TGTGTTGCAC AAAACACAAA TTCCACTGC 7849

15

(2) INFORMATION FOR SEQ ID NO:12:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1443 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGTCCTTGG CCCACCTCTC TCCCACCCGA CTCTGCCGAA AGAACACAG 50

30 AAGCTCAAGC CGCCTCCATG GCCCCAGGAA AGATTCAAGG GAGAGGCC 100

35 ATACAGGGAG CCACTTCACT TAGACACCCCT GGCCAGAATG GAGCTGACTG 150

ATTTGCTCCT GGCGGCCATG CTTCTTGCAG TGGCAAGACT AACTCTGTCC 200

40 AGCCCCGTAG CTCCCTGCCTG TGACCCAGA CTCCCTAAATA AACTGCTGCC 250

TGACTCCAC CTCCCTCACA GCCGACTGAG TCAGTGTCCC GACGTCGACC 300

45 CTTTGTCTAT CCCTGTTCTG CTGCCTGCTG TGGACTTTAG CCTGGGAGAA 350

50 TGGAAAACCC AGACGGAACA GAGCAAGGCA CAGGACATTC TAGGGCAGT 400

GTCCCTTCTA CTGGAGGGAG TGATGGCAGC ACGAGGACAG TTGGAACCC 450

55 CCTGCCTCTC ATCCCTCCTG GGACAGCTT CTGGGCAGGT TCGCCTCCTC 500

TTGGGGGCC TGCAGGGCCT CCTAGGAACC CAGGGCAGGA CCACAGCTCA 550

60 CAAGGACCCC AATGCCCTCT TCTTGAGCTT GCAACAACTG CTTGGGGAA 600

AGGTGGGCTT CCTGCTCTG GTAGAAGGTC CCACCCCTCG TGTCAGACGG 650  
5 ACCCTGCCAA CCACAGCTGT CCCAAGCAGT ACTTCTCAAC TCCTCACACT 700  
AAACAAGTTC CCAAACAGGA CTTCTGGATT GTTGGAGACG AACTTCAGTG 750  
10 TCACAGCCAG AACTGCTGGC CCTGGACTTC TGAGCAGGCT TCAGGGATTC 800  
15 AGAGTCAAGA TTACTCCTGG TCAGCTAAAT CAAACCTCCA GGTCCCCAGT 850  
CCAAATCTCT GGATACCTGA ACAGGACACA CGGACCTGTG AATGGAACTC 900  
20 ATGGGCTCTT TGCTGGAACC TCACTTCAGA CCCTGGAAGC CTCAGACATC 950  
TCGCCCCGAG CTTTCAACAA AGGCTCCCTG GCATTCAACC TCCAGGGTGG 1000  
25 ACTTCCTCCT TCTCCAAGCC TTGCTCCTGA TGGACACACA CCCTTCCCTC 1050  
30 CTTCACCTGC CTTGCCACC ACCCATGGAT CTCCACCCCC GCTCCACCCC 1100  
CTGTTTCCTG ACCCTTCCAC CACCATGCCT AACTCTACCG CCCCTCATCC 1150  
35 AGTCACAATG TACCCCTCATC CCAGGAATTT GTCTCAGGAA ACATAGCGGG 1200  
GGCACTGGCC CAGTGAGCGT CTGCAGCTTC TCTCGGGGAC AAGCTTCCCC 1250  
40 AGGAAGGCTG AGAGGCAGCT GCATCTGCTC CAGATGTTCT GCTTTCACCT 1300  
45 AAAAGGCCCT GGGGAAGGGA TACACAGCAC TGAGATTGT AAAATTTAG 1350  
GAGCTATTTT TTTTTAACCT ATCAGCAATA TTCATCAGAG CAGCTAGCGA 1400  
50 TCTTTGGTCT ATTTTCGGTA TAAATTGAA AATCACTAAT TCT 1443  
55 (2) INFORMATION FOR SEQ ID NO:13:  
60 (i) SEQUENCE CHARACTERISTICS:  
      (A) LENGTH: 352 amino acids  
      (B) TYPE: amino acid  
      (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

	Met	Glu	Leu	Thr	Asp	Leu	Leu	Leu	Ala	Ala	Met	Leu	Leu	Ala	Val	
5	1				5				10					15		
	Ala	Arg	Leu	Thr	Leu	Ser	Ser	Pro	Val	Ala	Pro	Ala	Cys	Asp	Pro	
					20				25					30		
10		Arg	Leu	Leu	Asn	Lys	Leu	Leu	Arg	Asp	Ser	His	Leu	Leu	His	Ser
					35				40					45		
	Arg	Leu	Ser	Gln	Cys	Pro	Asp	Val	Asp	Pro	Leu	Ser	Ile	Pro	Val	
					50				55					60		
15	Leu	Leu	Pro	Ala	Val	Asp	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln	
					65				70					75		
	Thr	Glu	Gln	Ser	Lys	Ala	Gln	Asp	Ile	Leu	Gly	Ala	Val	Ser	Leu	
20					80				85					90		
	Leu	Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln	Leu	Glu	Pro	Ser	
					95				100					105		
25	Cys	Leu	Ser	Ser	Leu	Leu	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	
					110				115					120		
	Leu	Leu	Gly	Ala	Leu	Gln	Gly	Leu	Leu	Gly	Thr	Gln	Gly	Arg	Thr	
					125				130					135		
30	Thr	Ala	His	Lys	Asp	Pro	Asn	Ala	Leu	Phe	Leu	Ser	Leu	Gln	Gln	
					140				145					150		
	Leu	Leu	Arg	Gly	Lys	Val	Arg	Phe	Leu	Leu	Leu	Val	Glu	Gly	Pro	
35					155				160					165		
	Thr	Leu	Cys	Val	Arg	Arg	Thr	Leu	Pro	Thr	Thr	Ala	Val	Pro	Ser	
					170				175					180		
40	Ser	Thr	Ser	Gln	Leu	Leu	Thr	Leu	Asn	Lys	Phe	Pro	Asn	Arg	Thr	
					185				190					195		
	Ser	Gly	Leu	Leu	Glu	Thr	Asn	Phe	Ser	Val	Thr	Ala	Arg	Thr	Ala	
					200				205					210		
45	Gly	Pro	Gly	Leu	Leu	Ser	Arg	Leu	Gln	Gly	Phe	Arg	Val	Lys	Ile	
					215				220					225		
	Thr	Pro	Gly	Gln	Leu	Asn	Gln	Thr	Ser	Arg	Ser	Pro	Val	Gln	Ile	
50					230				235					240		
	Ser	Gly	Tyr	Leu	Asn	Arg	Thr	His	Gly	Pro	Val	Asn	Gly	Thr	His	
					245				250					255		
55	Gly	Leu	Phe	Ala	Gly	Thr	Ser	Leu	Gln	Thr	Leu	Glu	Ala	Ser	Asp	
					260				265					270		
	Ile	Ser	Pro	Gly	Ala	Phe	Asn	Lys	Gly	Ser	Leu	Ala	Phe	Asn	Leu	
					275				280					285		
60	Gln	Gly	Gly	Leu	Pro	Pro	Ser	Pro	Ser	Leu	Ala	Pro	Asp	Gly	His	
					290				295					300		

Thr Pro Phe Pro Pro Ser Pro Ala Leu Pro Thr Thr His Gly Ser  
305 310 315

5 Pro Pro Gln Leu His Pro Leu Phe Pro Asp Pro Ser Thr Thr Met  
320 325 330

Pro Asn Ser Thr Ala Pro His Pro Val Thr Met Tyr Pro His Pro  
335 340 345

10 Arg Asn Leu Ser Gln Glu Thr  
350 352

(2) INFORMATION FOR SEQ ID NO:14:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1536 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
20 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 GAGTCCTTGG CCCACCTCTC TCCCACCCGA CTCTGCCGAA AGAAGCACAG 50

AAGCTCAAGC CGCCTCCATG GCCCCAGGAA AGATTCAAGG GAGAGGCC 100

30 ATACAGGGAG CCACCTTCAGT TAGACACCCT GGCCAGAACG GAGCTGACTG 150

35 ATTTGCTCCT GGCGGCCATG CTTCTTGCAG TGGCAAGACT AACTCTGTCC 200

AGCCCCGTAG CTCCTGCCTG TGACCCCAGA CTCCTAAATA AACTGCTGCG 250

40 TGACTCCCAC CTCCCTTCACA GCCGACTGAG TCAGTGTCCC GACGTCGACC 300

CTTTGTCTAT CCCTGTTCTG CTGCCCTGCTG TGGACTTTAG CCTGGGAGAA 350

45 TGGAAAACCC AGACGGAACA GAGCAAGGCA CAGGACATTC TAGGGGCAGT 400

50 GTCCCTTCTA CTGGAGGGAG TGATGGCAGC ACGAGGACAG TTGGAACCCT 450

CCTGCCTCTC ATCCCTCCTG GGACAGCTTT CTGGGCAGGT TCGCCTCCTC 500

55 TTGGGGGGCC TGCAGGGCCT CCTAGGAACC CAGCTTCCTC TACAGGGCAG 550

GACCACAGCT CACAAGGACC CCAATGCCCT CTTCTTGAGC TTGCAACAAAC 600

60 TGCTTCGGGG AAAGGTGCGC TTCCCTGCTTC TGGTAGAAGG TCCCACCCCTC 650

5 TGTGTCAGAC GGACCCGCC AACCACAGCT GTCCCAAGCA GTACTTCTCA 700  
ACTCCTCACA CTAAACAAGT TCCCAAACAG GACTTCTGGA TTGTTGGAGA 750

10 CGAACTTCAG TGTCACAGCC AGAACTGCTG GCCCTGGACT TCTGAGCAGG 800  
CTTCAGGGAT TCAGAGTCAA GATTACTCCT GGTCAAGCTAA ATCAAACCTC 850

15 CAGGTCCCCA GTCCAAATCT CTGGATACCT GAACAGGACA CACGGACCTG 900  
TGAATGGAAC TCATGGGCTC TTTGCTGGAA CCTCACTTCA GACCCTGGAA 950

20 GCCTCAGACA TCTCGCCCGG AGCTTTCAAC AAAGGCTCCC TGGCATTCAA 1000  
CCTCCAGGGT GGACTTCCTC CTTCTCCAAG CCTTGCTCCT GATGGACACA 1050

25 CACCCCTCCC TCCTTCACCT GCCTTGCCCA CCACCCATGG ATCTCCACCC 1100  
30 CAGCTCCACC CCCTGTTCC TGACCCCTCC ACCACCATGC CTAACCTCTAC 1150  
CGCCCCTCAT CCAGTCACAA TGTACCCCTCA TCCCAGGAAT TTGTCAGG 1200

35 AAACATAGCG CGGGCACTGG CCCAGTGAGC GTCTGCAGCT TCTCTCGGGG 1250  
ACAAGCTTCC CCAGGAAGGC TGAGAGGCAG CTGCATCTGC TCCAGATGTT 1300

40 CTGCTTTCAC CTAAAAGGCC CTGGGGAAAGG GATACACAGC ACTGGAGATT 1350  
45 GTAAAAATTTT AGGAGCTATT TTTTTTTAAC CTATCAGCAA TATTTCATCAG 1400  
AGCAGCTAGC GATCTTGGT CTATTTCTGG TATAAATTG AAAATCACTA 1450

50 AAAAAAAA AAAAAAAA AAAAAAAA AAAAAAAA AAAAAAAA 1500  
55 AAAAAAAA AAAAAAAA AAAAAAAA AAAAAA 1536

## (2) INFORMATION FOR SEQ ID NO:15:

60 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 356 amino acids

(B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

5	Met	Glu	Leu	Thr	Asp	Leu	Leu	Leu	Ala	Ala	Met	Leu	Leu	Ala	Val
	1			5				10			15				
10	Ala	Arg	Leu	Thr	Leu	Ser	Ser	Pro	Val	Ala	Pro	Ala	Cys	Asp	Pro
		20				25			30						
15	Arg	Leu	Leu	Asn	Lys	Leu	Leu	Arg	Asp	Ser	His	Leu	Leu	His	Ser
			35				40			45					
20	Arg	Leu	Ser	Gln	Cys	Pro	Asp	Val	Asp	Pro	Leu	Ser	Ile	Pro	Val
			50				55			60					
25	Leu	Leu	Pro	Ala	Val	Asp	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln
				65				70			75				
30	Thr	Glu	Gln	Ser	Lys	Ala	Gln	Asp	Ile	Leu	Gly	Ala	Val	Ser	Leu
				80				85			90				
35	Leu	Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln	Leu	Glu	Pro	Ser
				95				100			105				
40	Cys	Leu	Ser	Ser	Leu	Leu	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu
				110				115			120				
45	Leu	Leu	Gly	Ala	Leu	Gln	Gly	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Leu
				125				130			135				
50	Gln	Gly	Arg	Thr	Thr	Ala	His	Lys	Asp	Pro	Asn	Ala	Leu	Phe	Leu
				140				145			150				
55	Ser	Leu	Gln	Gln	Leu	Leu	Arg	Gly	Lys	Val	Arg	Phe	Leu	Leu	
				155				160			165				
60	Val	Glu	Gly	Pro	Thr	Leu	Cys	Val	Arg	Arg	Thr	Leu	Pro	Thr	Thr
				170				175			180				
65	Ala	Val	Pro	Ser	Ser	Thr	Ser	Gln	Leu	Leu	Thr	Leu	Asn	Lys	Phe
				185				190			195				
70	Pro	Asn	Arg	Thr	Ser	Gly	Leu	Leu	Glu	Thr	Asn	Phe	Ser	Val	Thr
				200				205			210				
75	Ala	Arg	Thr	Ala	Gly	Pro	Gly	Leu	Leu	Ser	Arg	Leu	Gln	Gly	Phe
				215				220			225				
80	Arg	Val	Lys	Ile	Thr	Pro	Gly	Gln	Leu	Asn	Gln	Thr	Ser	Arg	Ser
				230				235			240				
85	Pro	Val	Gln	Ile	Ser	Gly	Tyr	Leu	Asn	Arg	Thr	His	Gly	Pro	Val
				245				250			255				
90	Asn	Gly	Thr	His	Gly	Leu	Phe	Ala	Gly	Thr	Ser	Leu	Gln	Thr	Leu
				260				265			270				
95	Glu	Ala	Ser	Asp	Ile	Ser	Pro	Gly	Ala	Phe	Asn	Lys	Gly	Ser	Leu
				275				280			285				

	Ala Phe Asn Leu Gln Gly Gly Leu Pro Pro Ser Pro Ser Leu Ala		
	290	295	300
5	Pro Asp Gly His Thr Pro Phe Pro Pro Ser Pro Ala Leu Pro Thr		
	305	310	315
	Thr His Gly Ser Pro Pro Gln Leu His Pro Leu Phe Pro Asp Pro		
	320	325	330
10	Ser Thr Thr Met Pro Asn Ser Thr Ala Pro His Pro Val Thr Met		
	335	340	345
15	Tyr Pro His Pro Arg Asn Leu Ser Gln Glu Thr		
	350	355	356

## (2) INFORMATION FOR SEQ ID NO:16:

	(i) SEQUENCE CHARACTERISTICS:		
20	(A) LENGTH: 241 amino acids		
	(B) TYPE: amino acid		
	(D) TOPOLOGY: linear		
25	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:		
	Ser Pro Val Ala Pro Ala Cys Asp Pro Arg Leu Leu Asn Lys Leu		
	1	5	10
30	Leu Arg Asp Ser His Leu Leu His Ser Arg Leu Ser Gln Cys Pro		
	20	25	30
	Asp Val Asp Pro Leu Ser Ile Pro Val Leu Leu Pro Ala Val. Asp		
	35	40	45
35	Phe Ser Leu Gly Glu Trp Lys Thr Gln Thr Glu Gln Ser Lys Ala		
	50	55	60
	Gln Asp Ile Leu Gly Ala Val Ser Leu Leu Glu Gly Val Met		
40	65	70	75
	Ala Ala Arg Gly Gln Leu Glu Pro Ser Cys Leu Ser Ser Leu Leu		
	80	85	90
45	Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln		
	95	100	105
	Gly Leu Leu Gly Thr Gln Leu Pro Leu Gln Gly Arg Thr Thr Ala		
	110	115	120
50	His Lys Asp Pro Asn Ala Leu Phe Leu Ser Leu Gln Gln Leu Leu		
	125	130	135
	Arg Gly Lys Asp Phe Trp Ile Val Gly Asp Glu Leu Gln Cys His		
55	140	145	150
	Ser Gln Asn Cys Trp Pro Trp Thr Ser Glu Gln Ala Ser Gly Ile		
	155	160	165
60	Gln Ser Gln Asp Tyr Ser Trp Ser Ala Lys Ser Asn Leu Gln Val		
	170	175	180

	Pro	Ser	Pro	Asn	Leu	Trp	Ile	Pro	Glu	Gln	Asp	Thr	Arg	Thr	Cys
					185				190						195
5	Glu	Trp	Asn	Ser	Trp	Ala	Leu	Cys	Trp	Asn	Leu	Thr	Ser	Asp	Pro
					200				205						210
	Gly	Ser	Leu	Arg	His	Leu	Ala	Arg	Ser	Phe	Gln	Gln	Arg	Leu	Pro
					215				220						225
10	Gly	Ile	Gln	Pro	Pro	Gly	Trp	Thr	Ser	Ser	Phe	Ser	Lys	Pro	Cys
					230				235						240
	Ser														
15															
	241														

## (2) INFORMATION FOR SEQ ID NO:17:

	(i) SEQUENCE CHARACTERISTICS:														
	(A) LENGTH: 335 amino acids														
20	(B) TYPE: amino acid														
	(D) TOPOLOGY: linear														
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:														
25	Ser	Pro	Val	Ala	Pro	Ala	Cys	Asp	Pro	Arg	Leu	Leu	Asn	Lys	Leu
	1				5					10					15
	Leu	Arg	Asp	Ser	His	Leu	Leu	His	Ser	Arg	Leu	Ser	Gln	Cys	Pro
30					20				25						30
	Asp	Val	Asp	Pro	Leu	Ser	Ile	Pro	Val	Leu	Leu	Pro	Ala	Val	Asp
					35				40						45
35	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln	Thr	Glu	Gln	Ser	Lys	Ala
					50				55						60
	Gln	Asp	Ile	Leu	Gly	Ala	Val	Ser	Leu	Leu	Glu	Gly	Val	Met	
					65				70						75
40	Ala	Ala	Arg	Gly	Gln	Leu	Glu	Pro	Ser	Cys	Leu	Ser	Ser	Leu	Leu
					80				85						90
	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	Gly	Ala	Leu	Gln
45							95			100					105
	Gly	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Leu	Gln	Gly	Arg	Thr	Thr	Ala
					110				115						120
50	His	Lys	Asp	Pro	Asn	Ala	Leu	Phe	Leu	Ser	Leu	Gln	Gln	Leu	Leu
					125				130						135
	Arg	Gly	Lys	Val	Arg	Phe	Leu	Leu	Leu	Val	Glu	Gly	Pro	Thr	Leu
					140				145						150
55	Cys	Val	Arg	Arg	Thr	Ile	Pro	Thr	Thr	Ala	Val	Pro	Ser	Ser	Thr
					155				160						165
60	Ser	Gln	Leu	Leu	Thr	Leu	Asn	Lys	Phe	Pro	Asn	Arg	Thr	Ser	Gly
					170				175						180

	Leu Leu Glu Thr Asn Phe Ser Val Thr Ala Arg Thr Ala Gly Pro		
	185	190	195
5	Gly Leu Leu Ser Arg Leu Gln Gly Phe Arg Val Lys Ile Thr Pro		
	200	205	210
	Gly Gln Leu Asn Gln Thr Ser Arg Ser Pro Val Gln Ile Ser Gly		
	215	220	225
10	Tyr Leu Asn Arg Thr His Gly Pro Val Asn Gly Thr His Gly Leu		
	230	235	240
	Phe Ala Gly Thr Ser Leu Gln Thr Leu Glu Ala Ser Asp Ile Ser		
15	245	250	255
	Pro Gly Ala Phe Asn Lys Gly Ser Leu Ala Phe Asn Leu Gln Gly		
	260	265	270
20	Gly Leu Pro Pro Ser Pro Ser Leu Ala Pro Asp Gly His Thr Pro		
	275	280	285
	Phe Pro Pro Ser Pro Ala Leu Pro Thr Thr His Gly Ser Pro Pro		
	290	295	300
25	Gln Leu His Pro Leu Phe Pro Asp Pro Ser Thr Thr Met Pro Asn		
	305	310	315
	Ser Thr Ala Pro His Pro Val Thr Met Tyr Pro His Pro Arg Asn		
30	320	325	330
	Leu Ser Gln Glu Thr		
	335		
35	(2) INFORMATION FOR SEQ ID NO:18:		
	(i) SEQUENCE CHARACTERISTICS:		
	(A) LENGTH: 332 amino acids		
	(B) TYPE: amino acid		
	(D) TOPOLOGY: linear		
40	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:		
	Ser Pro Ala Pro Pro Ala Cys Asp Pro Arg Leu Leu Asn Lys Leu		
	1	5	10
45	15		
	Leu Arg Asp Ser His Val Leu His Gly Arg Leu Ser Gln Cys Pro		
	20	25	30
	Asp Ile Asn Pro Leu Ser Thr Pro Val Leu Leu Pro Ala Val Asp		
50	35	40	45
	Phe Thr Leu Gly Glu Trp Lys Thr Gln Thr Glu Gln Thr Lys Ala		
	50	55	60
55	Gln Asp Val Leu Gly Ala Thr Thr Leu Leu Leu Glu Ala Val Met		
	65	70	75
	Thr Ala Arg Gly Gln Val Gly Pro Pro Cys Leu Ser Ser Leu Leu		
	80	85	90
60			

	Val Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln		
	95	100	105
5	Asp Leu Leu Gly Met Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala		
	110	115	120
	His Lys Asp Pro Ser Ala Ile Phe Leu Asn Phe Gln Gln Leu Leu		
	125	130	135
10	Arg Gly Lys Val Arg Phe Leu Leu Leu Val Val Gly Pro Ser Leu		
	140	145	150
15	Cys Ala Lys Arg Ala Pro Pro Ala Ile Ala Val Pro Ser Ser Thr		
	155	160	165
	Ser Pro Phe His Thr Leu Asn Lys Leu Pro Asn Arg Thr Ser Gly		
	170	175	180
20	Leu Leu Glu Thr Asn Ser Ser Ile Ser Ala Arg Thr Thr Gly Ser		
	185	190	195
	Gly Phe Leu Lys Arg Leu Gln Ala Phe Arg Ala Lys Ile Pro Gly		
	200	205	210
25	Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly His		
	215	220	225
30	Gln Asn Gly Thr His Gly Pro Leu Ser Gly Ile His Gly Leu Phe		
	230	235	240
	Pro Gly Pro Gln Pro Gly Ala Leu Gly Ala Pro Asp Ile Pro Pro		
	245	250	255
35	Ala Thr Ser Gly Met Gly Ser Arg Pro Thr Tyr Leu Gln Pro Gly		
	260	265	270
	Glu Ser Pro Ser Pro Ala His Pro Ser Pro Gly Arg Tyr Thr Leu		
	275	280	285
40	Phe Ser Pro Ser Pro Thr Ser Pro Ser Pro Thr Val Gln Leu Gln		
	290	295	300
	Pro Leu Leu Pro Asp Pro Ser Ala Ile Thr Pro Asn Ser Thr Ser		
	305	310	315
45	Pro Leu Leu Phe Ala Ala His Pro His Phe Gln Asn Leu Ser Gln		
	320	325	330
50	Glu Glu		
	332		

## (2) INFORMATION FOR SEQ ID NO:19:

55 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1026 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCCCGGCTC CTCCCTGCCTG TGACCCCCGA CTCCCTAAATA AACTGCTTCG 50  
5 TGACTCCCAT GTCCTTCACG GCAGACTGAG CCAGTGCCCA GACATTAACC 100  
CTTTGTCCAC ACCTGTCCCTG CTGCCTGCTG TGGACTTCAC CTTGGGAGAA 150  
10 TGGAAAACCC AGACGGAGCA GACAAAGGCA CAGGATGTCC TGGGAGCCAC 200  
AACCCTTCTG CTGGAGGCAG TGATGACAGC ACGGGGACAA GTGGGACCCC 250  
15 CTTGCCTCTC ATCCCTGCTG GTGCAGCTTT CTGGACAGGT TCGCCTCCTC 300  
20 CTCGGGGCCC TGCAGGACCT CCTTGGAATG CAGCTTCCTC CACAGGAAAG 350  
GACCACAGCT CACAAGGATC CCAGTGCAT CTTCTGAAC TTCCAACAAC 400  
25 TGCTCCGAGG AAAGGTGCGT TTCTGCTCC TTGTAGTGGG GCCCTCCCTC 450  
TGTGCCAAGA GGGCCCCACC CGCCATAGCT GTCCCGAGCA GCACCTCTCC 500  
30 ATTCCACACA CTGAACAAGC TCCCAACAG GACCTCTGGA TTGTTGGAGA 550  
35 CAAACTCCAG TATCTCAGCC AGAACTACTG GCTCTGGATT TCTCAAGAGG 600  
CTGCAGGCAT TCAGAGCCAA GATTCTGGT CTGCTGAACC AAACCTCCAG 650  
40 GTCCCTAGAC CAAATCCCTG GACACCAGAA TGGGACACAC GGACCCCTGA 700  
GTGGAATTCA TGGACTCTTT CCTGGACCCC AACCCGGGGC CCTCGGAGCT 750  
45 CCAGACATTC CTCCAGCAAC TTCAGGCATG GGCTCCCGGC CAACCTACCT 800  
50 CCAGCCTGGA GAGTCTCCTT CCCCCAGCTCA CCCTTCTCCT GGACGATACA 850  
CTCTCTTCTC TCCTTCACCC ACCTCGCCCT CCCCCACAGT CCAGCTCCAG 900  
55 CCTCTGCTTC CTGACCCCTC TGCGATCACA CCCAACTCTA CCAGTCCTCT 950  
TCTATTGCA GCTCACCCCTC ATTTCCAGAA CCTGTCTCAG GAAGAGTAAG 1000  
60

GTGCTCAGAC CCTGCCAACT TCAGCA 1026

## 5 (2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1014 bases  
(B) TYPE: nucleic acid  
10 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

15 AGCCCGGCTC CTCCTGCCCTG TGACCCCCGA CTCCTAAATA AACTGCTTCG 50  
20 TGACTCCCAC GTCCTTCACG GCAGACTGAG CCAGTGCCCA GACATTAACC 100  
CTTTGTCCAC ACCTGTCCCTG CTGCCCTGCCCTG TGGACTTCAC CTTGGGAGAA 150  
25 TGGAAAACCC AGACGGAGCA GACAAAGGCA CAGGATGTCC TGGGAGCCAC 200  
AACCCCTTCTG CTGGAGGCAG TGATGACAGC ACGGGGACAA GTGGGACCCC 250  
30 CTTGCCCTCTC ATCCCTGCCCTG GTGCAGCTTT CTGGACAGGT TCGCCTCCCTC 300  
CTCGGGGCC CTCAGGACCT CCTTGAATG CAGGGAAGGA CCACAGCTCA 350  
35 CAAGGATCCC AGTGCCATCT TCCTGAACCTT CCAACAACTG CTCCGAGGAA 400  
40 AGGTGCGTTT CCTGCCCTTT GTAGTGGGGC CCTCCCTCTG TGCCAAGAGG 450  
GCCCCACCCG CCATAGCTGT CCCGAGCAGC ACCTCTCCAT TCCACACACT 500  
45 GAACAAGCTC CCAAACAGGA CCTCTGGATT GTTGGAGACA AACTCCAGTA 550  
50 TCTCAGGCCAG AACTACTGGC TCTGGATTT TCAAGAGGCT GCAGGCATTC 600  
AGAGCCAAGA TTCTGGTCT GCTGAACCAA ACCTCCAGGT CCCTAGACCA 650  
55 AATCCCTGGA CACCAGAATG GGACACACGG ACCCTTGAGT GGAATTCAATG 700  
GACTCTTCC TGGACCCCAA CCCGGGGCCC TCGGAGCTCC AGACATTCC 750  
60 CCAGCAACTT CAGGCATGGG CTCCCGGCCA ACCTACCTCC AGCCTGGAGA 800

GTCTCCTTCC CCAGCTCACC CTTCTCCTGG ACGATACACT CTCTTCTCTC 850  
 5 CTTCACCCAC CTCGCCCTCC CCCACAGTCC AGCTCCAGCC TCTGCTTCCT 900  
 10 GACCCCTCTG CGATCACACC CAACTCTACC AGTCCTCTTC TATTTGCAGC 950  
 TCACCCCTCAT TTCCAGAACCC TGTCTCAGGA AGAGTAAGGT GCTCAGACCC 1000  
 15 TGCCAACTTC AGCA 1014

20 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 328 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Pro	Arg	Leu	Leu	Asn	Lys	Leu
1					5				10				15	
Leu	Arg	Asp	Ser	His	Val	Leu	His	Gly	Arg	Leu	Ser	Gln	Cys	Pro
30					20				25				30	
Asp	Ile	Asn	Pro	Leu	Ser	Thr	Pro	Val	Leu	Leu	Pro	Ala	Val	Asp
35				35				40				45		
Phe	Thr	Leu	Gly	Glu	Trp	Lys	Thr	Gln	Thr	Glu	Gln	Thr	Lys	Ala
40				50				55				60		
Gln	Asp	Val	Leu	Gly	Ala	Thr	Thr	Leu	Leu	Leu	Glu	Ala	Val	Met
45				65				70				75		
Thr	Ala	Arg	Gly	Gln	Val	Gly	Pro	Pro	Cys	Leu	Ser	Ser	Leu	Leu
50				80				85				90		
Val	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	Gly	Ala	Leu	Gln
55				95				100				105		
Asp	Leu	Leu	Gly	Met	Gln	Gly	Arg	Thr	Thr	Ala	His	Lys	Asp	Pro
60				110				115				120		
Ser	Ala	Ile	Phe	Leu	Asn	Phe	Gln	Gln	Leu	Leu	Arg	Gly	Lys	Val
				125				130				135		
Arg	Phe	Leu	Leu	Leu	Val	Val	Gly	Pro	Ser	Leu	Cys	Ala	Lys	Arg
65				140				145				150		
Ala	Pro	Pro	Ala	Ile	Ala	Val	Pro	Ser	Ser	Thr	Ser	Pro	Phe	His
				155				160				165		

	Thr	Leu	Asn	Lys	Leu	Pro	Asn	Arg	Thr	Ser	Gly	Leu	Leu	Glu	Thr	
														170	175	180
5	Asn	Ser	Ser	Ile	Ser	Ala	Arg	Thr	Thr	Gly	Ser	Gly	Phe	Leu	Lys	
														185	190	195
	Arg	Leu	Gln	Ala	Phe	Arg	Ala	Lys	Ile	Pro	Gly	Leu	Leu	Asn	Gln	
														200	205	210
10	Thr	Ser	Arg	Ser	Leu	Asp	Gln	Ile	Pro	Gly	His	Gln	Asn	Gly	Thr	
														215	220	225
	His	Gly	Pro	Leu	Ser	Gly	Ile	His	Gly	Leu	Phe	Pro	Gly	Pro	Gln	
15														230	235	240
	Pro	Gly	Ala	Leu	Gly	Ala	Pro	Asp	Ile	Pro	Pro	Ala	Thr	Ser	Gly	
														245	250	255
20	Met	Gly	Ser	Arg	Pro	Thr	Tyr	Leu	Gln	Pro	Gly	Glu	Ser	Pro	Ser	
														260	265	270
	Pro	Ala	His	Pro	Ser	Pro	Gly	Arg	Tyr	Thr	Leu	Phe	Ser	Pro	Ser	
														275	280	285
25	Pro	Thr	Ser	Pro	Ser	Pro	Thr	Val	Gln	Leu	Gln	Pro	Leu	Leu	Pro	
														290	295	300
	Asp	Pro	Ser	Ala	Ile	Thr	Pro	Asn	Ser	Thr	Ser	Pro	Leu	Leu	Phe	
30														305	310	315
	Ala	Ala	His	Pro	His	Phe	Gln	Asn	Leu	Ser	Gln	Glu	Glu			
														320	325	328

(2) INFORMATION FOR SEQ ID NO:22:

35	(i) SEQUENCE CHARACTERISTICS:
	(A) LENGTH: 5141 bases
	(B) TYPE: nucleic acid
	(C) STRANDEDNESS: double
40	(D) TOPOLOGY: linear
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:
45	TTCGAGCTCG CCCGACATTG ATTATTGACT AGAGTCGATC GACAGCTGTG 50
	GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA 100
50	GAAGTATGCCA AAGCATGCAT CTCAATTAGT CAGCAACCAG GTGTGGAAAG 150
	TCCCCAGGCT CCCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA 200
55	GTCAGCAACC ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC 250
60	CGCCCAGTTC CGCCCATTCT CCGCCCCATG GCTGACTAAT TTTTTTTATT 300

TATGCAGAGG CCGAGGCCGC CTCGGCTCT GAGCTATTCC AGAAGTAGTG 350  
5 AGGAGGCTTT TTTGGAGGCC TAGGCTTTG CAAAAAGCTA GCTTATCCGG 400  
CGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAG AGTGACGTAA 450  
10 GTACCGCCTA TAGAGCGATA AGAGGATTTT ATCCCCGCTG CCATCATGGT 500  
TCGACCATTG AACTGCATCG TCGCCGTGTC CCAAAATATG GGGATTGGCA 550  
15 AGAACGGAGA CCTACCCCTGG CCTCCGCTCA GGAACGAGTT CAAGTACTTC 600  
20 CAAAGAATGA CCACAACCTC TTCAGTGGAA GGTAAACAGA ATCTGGTGAT 650  
TATGGGTAGG AAAACCTGGT TCTCCATTCC TGAGAAGAAT CGACCTTTAA 700  
25 AGGACAGAAAT TAATATAGTT CTCAGTAGAG AACTCAAAGA ACCACCACGA 750  
GGAGCTCATT TTCTTGCCAA AAGTTGGAT GATGCCTTAA GACTTATTGA 800  
30 ACAACCGGAA TTGGCAAGTA AAGTAGACAT GGTTGGATA GTCGGAGGCA 850  
35 GTTCTGTTTA CCAGGAAGCC ATGAATCAAC CAGGCCACCT TAGACTCTTT 900  
GTGACAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTT TCCCAGAAAT 950  
40 TGATTTGGGG AAATATAAAC CTCTCCCAGA ATACCCAGGC GTCTCTCTG 1000  
AGGTCCAGGA GGAAAAAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAAG 1050  
45 AAAGACTAAC AGGAAGATGC TTTCAAGTTC TCTGCTCCCC TCCTAAAGCT 1100  
50 ATGCATTTTT ATAAGACCAT GGGACTTTG CTGGCTTTAG ATCCCCCTGG 1150  
CTTCGTTAGA ACGCGGCTAC AATTAATACA TAACCTTATG TATCATAACAC 1200  
55 ATACGATTTA GGTGACACTA TAGATAACAT CCACCTTGCC TTTCTCTCCA 1250  
CAGGTGTCCA CTCCCAGGTC CAACTGCACC TCGGTTCTAA GCTTCTGCAG 1300  
60

GTCGACTCTA GAGGATCCCC GGGGAATTCA ATCGATGGCC GCCATGGCCC 1350  
5 AACTTGTATA TTGCAGCTTA TAATGGTTAC AAATAAAGCA ATAGCATCAC 1400  
AAATTCACA AATAAAGCAT TTTTTCACT GCATTCTAGT TGTGGTTTGT 1450  
10 CCAAACATCAT CAATGTATCT TATCATGTCT GGATCGATCG GGAATTAATT 1500  
CGGCGCAGCA CCATGGCCTG AAATAACCTC TGAAAGAGGA ACTTGGTTAG 1550  
15 GTACCTTCTG AGGCAGGAAAG AACCAGCTGT GGAATGTGTG TCAGTTAGGG 1600  
TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAAGCATGCA 1650  
20 TCTCAATTAG TCAGCAACCA GGTGTGAAA GTCCCCAGGC TCCCCAGCAG 1700  
GCAGAAGTAT GCAAAGCATG CATCTCAATT AGTCAGCAAC CATACTCCCG 1750  
CCCCTAACTC CGCCCATCCC GCCCCTAACT CCGCCCGAGTT CCGCCCATTC 1800  
30 TCCGCCCCAT GGCTGACTAA TTTTTTTTAT TTATGCAGAG GCCGAGGCCG 1850  
CCTCGGCCTC TGAGCTATTC CAGAAGTAGT GAGGAGGCTT TTTTGGAGGC 1900  
35 CTAGGCTTTT GCAAAAAGCT GTTACCTCGA GCGGCCGCTT AATTAAGGCG 1950  
40 CGCCATTTAA ATCCTGCAGG TAACAGCTTG GCACTGGCCG TCGTTTTACA 2000  
ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT CGCCTTGAG 2050  
45 CACATCCCCC CTTCGCCAGC TGGCGTAATA GCGAAGAGGC CCGCACCGAT 2100  
GCCCTTCCC AACAGTTGCG TAGCCTGAAT GGCGAATGGC GCCTGATGCG 2150  
50 GTATTTCTC CTTACGCATC TGTGCGGTAT TTCACACCCGC ATACGTCAA 2200  
GCAACCATAG TACCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG 2250  
TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT 2300  
60 CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG 2350

TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC 2400  
5 GGCACCTCGA CCCCCAAAAAA CTTGATTTGG GTGATGGTTC ACGTAGTGGG 2450  
10 CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT 2500  
CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAAACACTC AACCTATCT 2550  
15 CGGGCTATTTC TTTTGATTAA TAAGGGATTT TGCCGATTTTC GGCCTATTGG 2600  
TTAAAAAAATG AGCTGATTAA ACAAAAATTT AACCGGAATT TTAACAAAAT 2650  
20 ATTAACGTTT ACAATTATAT GGTGCACTCT CAGTACAATC TGCTCTGATG 2700  
25 CCGCATAGTT AAGCCAACTC CGCTATCGCT ACGTGACTGG GTCATGGCTG 2750  
CGCCCCGACA CCCGCCAACCA CCCGCTGACG CGCCCTGACG GGCTTGTCTG 2800  
30 CTCCCCGCAT CCGCTTACAG ACAAGCTGTG ACCGTCTCCG GGAGCTGCAT 2850  
GTGTCAGAGG TTTTCACCGT CATCACCGAA ACGCGCGAGG CAGTATTCTT 2900  
35 GAAGACGAAA GGGCCTCGTG ATACGCCAT TTTTATAGGT TAATGTCATG 2950  
ATAATAATGG TTTCTTAGAC GTCAGGTGGC ACTTTTCGGG GAAATGTGCG 3000  
40 CGGAACCCCT ATTTGTTTAT TTTTCTAAAT ACATTCAAAT ATGTATCCGC 3050  
45 TCATGAGACA ATAACCCCTGA TAAATGCTTC AATAATATTG AAAAAGGAAG 3100  
AGTATGAGTA TTCAACATTT CCGTGTGCC CTTATTCCCT TTTTGCGGC 3150  
50 ATTTTGCCCT CCTGTTTTG CTCACCCAGA AACGCTGGTG AAAGTAAAAG 3200  
ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA ACTGGATCTC 3250  
55 AACAGCGGTA AGATCCTTGA GAGTTTCGC CCCGAAGAAC GTTTTCCAAT 3300  
60 GATGAGCACT TTTAAAGTTC TGCTATGTGG CGCGGTATTA TCCC GTGATG 3350

ACGCCGGGCA AGAGCAACTC GGTCGCCGCA TACACTATTG TCAGAATGAC 3400  
5 TTGGTTGAGT ACTCACCACTG CACAGAAAAG CATCTTACGG ATGGCATGAC 3450  
AGTAAGAGAA TTATGCAGTG CTGCCATAAC CATGAGTGAT AACACTGCGG 3500  
10 CCAACTTACT TCTGACAACG ATCGGAGGAC CGAAGGAGCT AACCGCTTT 3550  
TTGCACAAACA TGGGGGATCA TGTAACTCGC CTTGATCGTT GGGAACCGGA 3600  
15 GCTGAATGAA GCCATACCAA ACGACGAGCG TGACACCACG ATGCCAGCAG 3650  
20 CAATGGCAAC AACGTTGGCG AACACTATTAA CTGGCGAACT ACTTACTCTA 3700  
GCTTCCCGGC AACAAATTAAAT AGACTGGATG GAGGCGGATA AAGTTGCAGG 3750  
25 ACCACTTCTG CGCTCGGGCC TTCCGGCTGG CTGGTTTATT GCTGATAAAAT 3800  
CTGGAGCCGG TGAGCGTGGG TCTCGCGGT A TCATTGCAGC ACTGGGGCCA 3850  
30 GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC 3900  
35 AACTATGGAT GAACGAAATA GACAGATCGC TGAGATAGGT GCCTCACTGA 3950  
TTAAGCATTG GTAACGTCA GACCAAGTTT ACTCATATAT ACTTTAGATT 4000  
40 GATTTAAAAC TTCATTTTA ATTTAAAAGG ATCTAGGTGA AGATCCTTTT 4050  
TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTCG TTCCACTGAG 4100  
45 CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA TCCTTTTTT 4150  
50 CTGCGCGTAA TCTGCTGCTT GCAAAACAAA AAACCACCGC TACCAGCGGT 4200  
GGTTTGTGTTG CGGGATCAAG AGCTACCAAC TCTTTTCCG AAGGTAACTG 4250  
55 GCTTCAGCAG AGCGCAGATA CCAAATACTG TCCTTCTAGT GTAGCCGTAG 4300  
TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT ACCTCGCTCT 4350  
60

GCTAATCCTG TTACCAGTGG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA 4400

5 CCGGGTTGGA CTCAGACGA TAGTTACCGG ATAAGGCAGA GCGGTCGGGC 4450

TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA CGACCTACAC 4500

10 CGAACTGAGA TACCTACAGC GTGAGCATTG AGAAAGCGCC ACGCTTCCCG 4550

AAGGGAGAAA GGCAGGACAGG TATCCGTAA GCGGCAGGGT CGGAACAGGA 4600

15 GAGCGCACGA GGGAGCTTCC AGGGGAAAC GCCTGGTATC TTTATAGTCC 4650

20 TGTGGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTG TGATGCTCGT 4700

CAGGGGGGGG GAGCCTATGG AAAAACGCCA GCAACCGGGC CTTTTTACGG 4750

25 TTCCCTGGCCT TTTGCTGCC TTTGCTCAC ATGTTCTTC CTGCGTTATC 4800

CCCTGATTCT GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATAACCG 4850

30 CTCGCCGCAG CGAACGACC GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG 4900

35 GAAGAGCGCC CAATACGCAA ACCGCCTCTC CCCGCGCGTT GGCGATTCA 4950

TTAATCCAGC TGGCACGACA GGTTTCCCGA CTGGAAAGCG GGCAGTGAGC 5000

40 GCAACGCAAT TAATGTGAGT TACCTCACTC ATTAGGCACC CCAGGCTTTA 5050

CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA 5100

45 ATTCACACA GGAAACAGCT ATGACCATGA TTACGAATTAA 5141

50 (2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

60 ATGTCNCCNG CNCCNCCNGC N 21

## (2) INFORMATION FOR SEQ ID NO:24:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 bases  
(B) TYPE: nucleic acid  
10 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

15 ATGTCTCCAG CGCCGCCAGC G 21

## (2) INFORMATION FOR SEQ ID NO:25:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 bases  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

30 ATGTCGCCTG CTCCACCTGC T 21

## (2) INFORMATION FOR SEQ ID NO:26:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 bases  
(B) TYPE: nucleic acid  
40 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

45 ATGTCGCCAG CGCCACCAGC C 21

## (2) INFORMATION FOR SEQ ID NO:27:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 bases  
(B) TYPE: nucleic acid  
55 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

60 ATGTCCCCAG CCCCCACCCGC A 21

## (2) INFORMATION FOR SEQ ID NO:28:

## 5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

15 ATGTCGCCAG CGCCGCCAGC G 21

## (2) INFORMATION FOR SEQ ID NO:29:

## 20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Pro	Arg	Leu	Leu	Asn	Lys	Leu
1					5			10					15	

30 Leu Arg Asp Asp His Val Leu His Gly Arg  
20 25

## (2) INFORMATION FOR SEQ ID NO:30:

## 35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Pro	Arg	Leu	Leu	Asn	Lys	Leu
1					5			10					15	

45 Leu Arg Asp Asp Xaa Val Leu His Gly Arg Leu  
20 25 26

## (2) INFORMATION FOR SEQ ID NO:31:

## 50 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## 55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ser	Pro	Ala	Pro	Pro	Ala	Xaa	Asp	Pro	Arg	Leu	Leu	Asn	Lys	Leu
1					5			10					15	

60 Leu Arg Asp Asp His Val Leu His Gly Arg  
20 25

## (2) INFORMATION FOR SEQ ID NO:32:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Xaa Pro Ala Pro Pro Ala Xaa Asp Pro Arg Leu Xaa Asn Lys  
1 5 10 14

## 15 (2) INFORMATION FOR SEQ ID NO:33:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Pro Arg Leu Leu Asn Lys Leu Leu Arg  
1 5 9

## 25 (2) INFORMATION FOR SEQ ID NO:34:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCCGTGAAGG ACGTGGTCGT CACGAAGCAG TTTATTTAGG AGTCG 45

## 40 (2) INFORMATION FOR SEQ ID NO:35:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCNGCNCCNC CNGCNTGYGA 20

## 55 (2) INFORMATION FOR SEQ ID NO:36:

60 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

5

NCCRTGNARN ACRTGRTCRT C 21

10 (2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

20

CCAGCGCCGC CAGCCTGTGA CCCCCGACTC CTAAATAAAC TGCCCTCGTGA 50

25

TGACCACGTT CAGCACGGC 69

(2) INFORMATION FOR SEQ ID NO:38:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

40

GGTCGCGGGCG GTCGGACACT GGGGGCTGAG GATTTATTTG ACGGAGCACT 50

ACTGGTGCAA GTCGTGCCG 69

45

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCAGCACCTC CGGCATGTGA CCCCCGACTC CTAAATAAAC TGCTTCGTGA 50

60

CGACCACGTC CATCACGGC 69

## (2) INFORMATION FOR SEQ ID NO:40:

## 5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

15 GGTCTGTGGAG GCCGTACACT GGGGGCTGAG GATTTATTTG ACGAAGCACT 50

GCTGGTGCAG GTAGTGCCG 69

20 (2) INFORMATION FOR SEQ ID NO:41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

30 CCAGCACCGC CGGCATGTGA CCCCCGACTC CTAATAAAC TGCTTCGTGA 50

35 CGATCATGTC TATCACGGT 69

40 (2) INFORMATION FOR SEQ ID NO:42:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

50 GGTCTGGCG GCCGTACACT GGGGGCTGAG GATTTATTTG ACGAAGCACT 50

55 GCTAGTACAG ATAGTGCCA 69

55 (2) INFORMATION FOR SEQ ID NO:43:

## (i) SEQUENCE CHARACTERISTICS:

60 

- (A) LENGTH: 37 bases
- (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCTAGCTCTA GAAATTGCTC CTCGTGGTCA TGCTTCT 37

10 (2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

20 CAGTCTGCCG TGAAGGACAT GG 22

25 (2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

35 TGTGGACTTT AGCTTGGGAG AATG 24

40 (2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

50 GGTCCAGGGA CCTGGAGGTT TG 22

55 (2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 31 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

5

ATCGATATCG ATAGCCAGAC ACCCCGGCCA G 31

10 (2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

20

GCTAGCTCTA GACAGGGAAG GGAGCTGTAC ATGAGA 36

25 (2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

35

CTCCTTGGAA CCCAGGGCAG GACC 24

40 (2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

50

GGTCCTGCC TGGGTTCCAA GGAG 24

55 (2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

5 CTGCTCCGAG GAAAGGACTT CTGGATT 27

(2) INFORMATION FOR SEQ ID NO:52:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

20 AATCCAGAAG TCCTTTCTTC GGAGCAG 27

(2) INFORMATION FOR SEQ ID NO:53:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
30 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

35 CCCTCTGCGT CGCGGCGGCC CCACCCAC 28

(2) INFORMATION FOR SEQ ID NO:54:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
45 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

50 GTGGGTGGGG CGGCCGCGAC GCAGAGGG 28

(2) INFORMATION FOR SEQ ID NO:55:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
60 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

5 GACTCGAGGA TCCATCGATT TTTTTTTTTT TTTTT 35

(2) INFORMATION FOR SEQ ID NO:56:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

20 GACTCGAGGA TCCATCG 17

(2) INFORMATION FOR SEQ ID NO:57:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

35 GCTAGCTCTA GAAGCCCGGC TCCTCCTGCC TG 32

(2) INFORMATION FOR SEQ ID NO:58:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

50 CGAAATTAAC CCTCACTAAA G 21

(2) INFORMATION FOR SEQ ID NO:59:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 103 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

1 TGCAGCAAGG GCTACTGCCA CACTCGAGCT GCGCAGATGC TAGCCTCAAG 50

5 ATGGCTGATC GAAATCGATT CCGCGGCAAA GATCTTCCGG TCCTGTAGAA 100

10 GCT 103

(2) INFORMATION FOR SEQ ID NO:60:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 103 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

25 AGCTTCTACA GGACCGGAAG ATCTTGCCG CGGAATCGAT TTGGATCAGC 50

CATCTTGAGG CTAGCATCTG CGCAGCTCGA GTGTGGCAGT AGCCCTTGCT 100

30 GCA 103

(2) INFORMATION FOR SEQ ID NO:61:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

45 TCTCGCTACC GTTTACAG 18

50 (2) INFORMATION FOR SEQ ID NO:62:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

60 CAGGTACCCA CCAGGCGGTC TCGGT 25

## (2) INFORMATION FOR SEQ ID NO:63:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

15 GGGCCATGAC ACTGTCAA 18

## (2) INFORMATION FOR SEQ ID NO:64:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 40 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

30 GACCGCCACC GAGACCGCCT GGTGGGTACC TGTGGTCCTT 40

## (2) INFORMATION FOR SEQ ID NO:65:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

45 ATCGATATCG ATCAGCCAGA CACCCCGGCC AG 32

## (2) INFORMATION FOR SEQ ID NO:66:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

60 TCTAGATCTA GATCACCTGA CGCAGAGGGT GGACC 35

## (2) INFORMATION FOR SEQ ID NO:67:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

AGTCGACGTC GACGTCGGCA GTGTCTGAGA ACC 33

15

## (2) INFORMATION FOR SEQ ID NO:68:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

AGTCGACGTC GACTCACCTG ACGCAGAGGG TGGACC 36

30

## (2) INFORMATION FOR SEQ ID NO:69:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 62 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

CGCGTATGCC AGCCCGGCTC CTCCTGCTTG TGACCTCCGA GTCCTCAGTA 50

45

AACTGCTTCG TG 62

50 (2) INFORMATION FOR SEQ ID NO:70:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 61 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

60

ATACGGTCGG GCCGAGGAGG ACGAACACTG GAGGCTCAGG AGTCATTTGA 50

CGAAGCACTG A 61

5

(2) INFORMATION FOR SEQ ID NO:71:

10

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC TTCTTAA 37

20

(2) INFORMATION FOR SEQ ID NO:72:

25

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

TTAATACATT TTCTTATAGC GTAAAGAAGA ATTGCGC 37

35

(2) INFORMATION FOR SEQ ID NO:73:

40

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 69 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC ATCACCATCA CCATCACCAT 50

50

CACATCGAAG GTCGTAGCC 69

55 (2) INFORMATION FOR SEQ ID NO:74:

60

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 62 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

5 TTAATACTTT TTCTTATAGC GTAAAGTAGT GGTAGTGGTA GTGGTAGTGT 50

AGCTTCCAGC AT 62

10

(2) INFORMATION FOR SEQ ID NO:75:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC ATCACCATCA CCATCACCAT 50

25

CACATCGAAC CACGTAGCC 69

30 (2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

40

TTAATACTTT TTCTTATAGC GTAAAGTAGT GGTAGTGGTA GTGGTAGTGT 50

45

AGCTTGGTGC AT 62

(2) INFORMATION FOR SEQ ID NO:77:

50 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

TCCACCCCTCT GCGTCAGGT 19

60

## (2) INFORMATION FOR SEQ ID NO:78:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GGAGACGCAG TCCATCGA 18

15

## (2) INFORMATION FOR SEQ ID NO:79:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 62 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

GCAGCAGTTC TAGAATTATG TCNCCNGCNC CNCCNGCNTG TGACCTCCGA 50

30

GTTCTCAGTA AA 62

35 (2) INFORMATION FOR SEQ ID NO:80:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 49 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

45

ACACTGGAGG CTCAAGAGTC ATTTGACGAA GCACTGAGGG TACAGGAAG 49

50 (2) INFORMATION FOR SEQ ID NO:81:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

60

CTAGAATTAT GAAAAAGAAT ATCGCATTAA TCGAAGGTCG TAGCC 45

## (2) INFORMATION FOR SEQ ID NO:82:

5

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
10 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

15 TTAATAC TTT TTCTTATAGC GTAAATAGCT TCCAGCAT 38

## (2) INFORMATION FOR SEQ ID NO:83:

20

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

30 CTAGAATTAT GAAAAAGAAT ATCGCATTTC TTCTTAAACG TAGCC 45

## (2) INFORMATION FOR SEQ ID NO:84:

35

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
40 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

45 TTAATAC TTT TTCTTATAGC GTAAAGAAGA ATTTGCAT 38

## (2) INFORMATION FOR SEQ ID NO:85:

50

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
55 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Met Lys Lys Asn Ile Ala Phe Leu Leu Asn Ala Tyr Ala Ser Pro  
1 5 10 15

60

Ala Pro Pro Ala Cys Cys Val Arg Arg Ala  
20 25

5 (2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Met Lys Lys Asn Ile Ala Phe His His His His His His His  
1 5 10 15

15 Ile Glu Gly Arg Ser Pro Ala Pro Pro Ala Cys Cys Val Arg Arg  
20 25 30

20 Ala  
31

25 (2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Met Lys Lys Asn Ile Ala Phe His His His His His His His  
1 5 10 15

35 Ile Glu Pro Arg Ser Pro Ala Pro Pro Ala Cys Cys Val Arg Arg  
20 25 30

Ala  
31

40 (2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Thr Thr Ala His Lys Asp Pro  
50 1 5 7

55 (2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

His Val Leu His  
1 4

## (2) INFORMATION FOR SEQ ID NO:90:

5

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

Ser Arg Leu Ser  
1 4

15

## (2) INFORMATION FOR SEQ ID NO:91:

20

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

25

Ser His Val Leu  
1 4

## (2) INFORMATION FOR SEQ ID NO:92:

30

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

His Ser Arg Leu  
1 4

40

## (2) INFORMATION FOR SEQ ID NO:93:

45

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

50

Ala Val Asp Phe  
1 4

## (2) INFORMATION FOR SEQ ID NO:94:

55

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

5 Ser Leu Gly Glu  
1 4

## (2) INFORMATION FOR SEQ ID NO:95:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

Ala Val Thr Leu  
1 4

## 15 (2) INFORMATION FOR SEQ ID NO:96:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

Leu Leu Glu Gly  
1 4

## 25 (2) INFORMATION FOR SEQ ID NO:97:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

Leu Ser Ser Leu  
1 4

## 40 (2) INFORMATION FOR SEQ ID NO:98:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

Leu Gly Gln Leu  
1 4

## 55 (2) INFORMATION FOR SEQ ID NO:99:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

5  
Cys Xaa Leu Ser Ser  
1 5

10 (2) INFORMATION FOR SEQ ID NO:100:

5 (i) SEQUENCE CHARACTERISTICS:  
1 (A) LENGTH: 4 amino acids  
10 (B) TYPE: amino acid  
15 (C) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

15 Leu Leu Gly Gln  
1 4

20 (2) INFORMATION FOR SEQ ID NO:101:

15 (i) SEQUENCE CHARACTERISTICS:  
20 (A) LENGTH: 4 amino acids  
25 (B) TYPE: amino acid  
30 (C) TOPOLOGY: linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

25 Ser Ser Leu Leu  
1 4

35 (2) INFORMATION FOR SEQ ID NO:102:

30 (i) SEQUENCE CHARACTERISTICS:  
35 (A) LENGTH: 4 amino acids  
40 (B) TYPE: amino acid  
45 (C) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

50 Gly Gln Leu Ser  
1 4

55 (2) INFORMATION FOR SEQ ID NO:103:

50 (i) SEQUENCE CHARACTERISTICS:  
55 (A) LENGTH: 4 amino acids  
60 (B) TYPE: amino acid  
65 (C) TOPOLOGY: linear

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

70 Cys Leu Ser Ser  
1 4

75 (2) INFORMATION FOR SEQ ID NO:104:

70 (i) SEQUENCE CHARACTERISTICS:  
75 (A) LENGTH: 4 amino acids  
80 (B) TYPE: amino acid  
85 (C) TOPOLOGY: linear

85 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

Leu Gln Ser Leu  
1 4

5 (2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

Leu Gly Thr Gln  
1 4

15 (2) INFORMATION FOR SEQ ID NO:106:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

25 Ala Leu Gln Ser  
1 4

(2) INFORMATION FOR SEQ ID NO:107:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

Leu Leu Gly Thr  
1 4

40 (2) INFORMATION FOR SEQ ID NO:108:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
45 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

50 Asn Ala Ile Phe  
1 4

(2) INFORMATION FOR SEQ ID NO:109:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

60

Leu Ser Phe Gln  
1 4

5 (2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu  
1 5 10 15  
15 Leu Arg Asp Ser His Val Leu  
20 22

20 (2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr  
1 5 10 15  
30 Pro Val Leu Leu Pro Ala Val Asp Phe  
20 24

35 (2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln  
1 5 10 15  
45 Asp Ile Leu Gly Ala Val Thr Leu  
20 23

50 (2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr  
1 5 10 15  
60

Cys Leu Ser Ser Leu Leu  
20 21

5 (2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln  
1 5 10 15

15 Ser  
16

20 (2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His  
1 5 10 15

30 Lys Asp Pro Asn Ala Ile Phe  
20 22

35 (2) INFORMATION FOR SEQ ID NO:116:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met  
1 5 10 15

45 Leu Val Gly Gly Ser Thr Leu Cys Val Arg  
20 25

50 (2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

Met Pro Pro Ala  
1 4

60

## (2) INFORMATION FOR SEQ ID NO:118:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

10 Met Ala Pro Pro Ala  
1 5

## (2) INFORMATION FOR SEQ ID NO:119:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

Met Pro Ala Pro Pro Ala  
1 5 6

## 25 (2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
30 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

35 Met Ser Pro Ala Pro Pro Ala  
1 5 7

## (2) INFORMATION FOR SEQ ID NO:121:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Ala Pro Pro Ala  
1 4

## (2) INFORMATION FOR SEQ ID NO:122:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

Pro Ala Pro Pro Ala  
1 5

60

## (2) INFORMATION FOR SEQ ID NO:123:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

10 Ser Pro Ala Pro Pro Ala  
1 5 6

:

## (2) INFORMATION FOR SEQ ID NO:124:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

Val Arg Arg Ala  
1 4

## 25 (2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
30 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

35 Val Arg Arg Ala Pro  
1 5

## (2) INFORMATION FOR SEQ ID NO:126:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

45 Val Arg Arg Ala Pro Pro  
1 5 6

## (2) INFORMATION FOR SEQ ID NO:127:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

Val Arg Arg Ala Pro Pro Thr  
1 5 7

60

## (2) INFORMATION FOR SEQ ID NO:128:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

10 Val Arg Arg Ala Pro Pro Thr Thr  
1 5 8

## (2) INFORMATION FOR SEQ ID NO:129:

## 15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

Val Arg Arg Ala Pro Pro Thr Thr Ala  
1 5 9

## 25 (2) INFORMATION FOR SEQ ID NO:130:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

35 Val Arg Arg Ala Pro Pro Thr Thr Ala Val  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:131:

## 40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro  
1 5 10 11

## 50 (2) INFORMATION FOR SEQ ID NO:132:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

55

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser  
1 5 10 12

60

## (2) INFORMATION FOR SEQ ID NO:133:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 13 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

10 Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg  
1 5 10 13

:

## (2) INFORMATION FOR SEQ ID NO:134:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr  
1 5 10 14

## 25 (2) INFORMATION FOR SEQ ID NO:135:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

35 Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:136:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser  
1 5 10 15

50 Leu  
16

## (2) INFORMATION FOR SEQ ID NO:137:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

:

## 60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

MPAPPA, (SEQ ID NO: 119)  
MSPAPPA, (SEQ ID NO: 120)

A,

PA,

PPA,

APPA, (SEQ ID NO: 121)

PAPPA, (SEQ ID NO: 122)

SPAPPA, (SEQ ID NO: 123)

Y represents the carboxy terminal group of Cys<sup>151</sup> or carboxy-terminus amino acid residue(s) selected from the group

V,

VR,

VRR,

VRRA, (SEQ ID NO: 124)

VRRAP, (SEQ ID NO: 125)

VRRAPP, (SEQ ID NO: 126)

VRRAPPT, (SEQ ID NO: 127)

VRRAPPTT, (SEQ ID NO: 128)

VRRAPPTTA, (SEQ ID NO: 129)

VRRAPPTTAV, (SEQ ID NO: 130)

VRRAPPTTAVP, (SEQ ID NO: 131)

VRRAPPTTAVPS, (SEQ ID NO: 132)

VRRAPPTTAVPSR, (SEQ ID NO: 133)

VRRAPPTTAVPSRT, (SEQ ID NO: 134)

VRRAPPTTAVPSRTS, (SEQ ID NO: 135)

VRRAPPTTAVPSRTSL, (SEQ ID NO: 136)

VRRAPPTTAVPSRTSLV, (SEQ ID NO: 137)

VRRAPPTTAVPSRTSLVL, (SEQ ID NO: 138)

VRRAPPTTAVPSRTSLVLT, (SEQ ID NO: 139)

VRRAPPTTAVPSRTSLVLT, (SEQ ID NO: 140)

VRRAPPTTAVPSRTSLVLTLN, (SEQ ID NO: 141)

VRRAPPTTAVPSRTSLVLTNE, (SEQ ID NO: 142)

VRRAPPTTAVPSRTSLVLTNEL, (SEQ ID NO: 143)

VRRAPPTTAVPSRTSLVLTNEP, (SEQ ID NO: 144),

amino-terminus amino acid residue extensions comprising one or more of the residues 176-332 of human ML as provided in Fig. 1 (SEQ ID NO: 1) and pegylated forms thereof.

7. A fragment polypeptide according to Claim 6 selected from the group TPO(1-153) and TPO(1-245).
- 5 8. A fragment polypeptide according to Claim 2, wherein the amino acid sequence of the fragment polypeptide comprises  
SPAPPACDLRVLSKLLRDSHVL,  
(SEQ ID NO: 110)  
HSRLSQCPEVHPLPTPVLLPAVDF,  
10 (SEQ ID NO: 111)  
SLGEWKTQMEETKAQDILGAVTL,  
(SEQ ID NO: 112)  
LLEGVMAARGQLGPTCLSSLL,  
(SEQ ID NO: 113)  
15 GQLSGQVRLLL GALQS,  
(SEQ ID NO: 114)  
LLGTQLPPQGRRTAHKDPNAIF,  
(SEQ ID NO: 115)  
LSFQHLLRGKVRFLMLVGGSTLCVR, and  
20 (SEQ ID NO: 116)  
combinations thereof.
9. The polypeptide of Claim 6 that is unglycosylated.
- 25 10. An isolated polypeptide encoded by a nucleic acid having a sequence that hybridizes under moderately stringent conditions to the nucleic acid molecules having a nucleic acid sequence provided in Fig. 1 (SEQ ID NO: 2).
11. The polypeptide of Claim 11 that is biologically active.
- 30 12. The polypeptide of Claim 1 selected from the group hML, hML153, hML(R153A, R154A), hML2, hML3, hML4, mML, mML2, mML3, pML, and pML2.
- 35 13. A polypeptide according to Claim 2, wherein the amino acid sequence of the polypeptide comprises amino acid residues 1 to X of Fig. 1 (SEQ ID NO: 1), where X is selected from the group 153, 155, 164, 174, 191, 205, 207, 217, 229, 245 and 332.

14. An isolated substantially homogeneous *mpl* ligand polypeptide sharing at least 80% sequence identity with the polypeptide of Claim 13.
- 5 15. The polypeptide of Claim 13 wherein X is 153.
16. A chimera comprising the *mpl* ligand of Claim 13 fused to a heterologous polypeptide.
- 10 17. The chimera of Claim 16 wherein the heterologous polypeptide is an immunoglobulin polypeptide.
18. The chimera of Claim 16 wherein the heterologous polypeptide is an interleukin polypeptide.
- 15 19. A chimera comprising the N-terminus residues 1 to about 153 to 157 of hML substituted with one or more, but not all, of the human EPO residues added or substituted into the N-terminus residues of hML at positions corresponding to the alignment shown in Fig. 10.
- 20 20. An antibody that is capable of binding the *mpl* ligand polypeptide of Claim 13.
21. A hybridoma cell line producing the antibody of Claim 20.
- 25 22. An isolated nucleic acid molecule encoding the *mpl* ligand polypeptide of Claim 1.
23. An isolated nucleic acid molecule encoding the *mpl* ligand polypeptide of Claim 13.
- 30 24. An isolated nucleic acid molecule comprising the open reading frame nucleic acid sequence shown in Fig. 1 (SEQ ID NO: 2).
- 25 35 25. The isolated nucleic acid molecule of Claim 24 encoding a *mpl* ligand polypeptide selected from the group hML, hML153, hML(R153A, R154A), hML2, hML3, hML4, mML, mML2, mML3, pML, and pML2.

26. An isolated nucleic acid molecule selected from the group consisting of
  - (a) a cDNA clone comprising the nucleotide sequence of the coding region of the *mpl* ligand gene;
  - (b) a DNA sequence capable of hybridizing under stringent conditions to a clone of (a); and
  - (c) a genetic variant of any of the DNA sequences of (a) and (b) which encodes a polypeptide possessing a biological property of a naturally occurring *mpl* ligand polypeptide.
- 10 27. An isolated DNA molecule having a sequence capable of hybridizing to a DNA sequence provided in Fig. 1 (SEQ ID NO: 2) under moderately stringent conditions, wherein the DNA molecule encodes a biologically active *mpl* ligand polypeptide.
- 15 28. The nucleic acid molecule of Claim 25 further comprising a promoter operably linked to the nucleic acid molecule.
29. An expression vector comprising the nucleic acid sequence of Claim 25 operably linked to control sequences recognized by a host cell transformed with the vector.
- 20 30. A host cell transformed with the vector of Claim 29.
31. A process of using a nucleic acid molecule encoding the *mpl* ligand polypeptide to effect production of the *mpl* ligand polypeptide comprising culturing the host cell of Claim 30.
- 25 32. The process of Claim 31 wherein the *mpl* ligand polypeptide is recovered from the host cell.
- 30 33. The process of Claim 31 wherein the *mpl* ligand polypeptide is recovered from the host cell culture medium.
- 35 34. A method of determining the presence of *mpl* ligand polypeptide, comprising hybridizing DNA encoding the *mpl* ligand polypeptide to a test sample nucleic acid and determining the presence of *mpl* ligand polypeptide DNA.

35. A method of amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase reaction with nucleic acid encoding a *mpl* ligand polypeptide.
- 5 36. A composition comprising the *mpl* ligand polypeptide of Claim 1 and a pharmaceutically acceptable carrier.
- 10 37. A method for treating a mammal having or at risk for thrombocytopenia comprising administering to a mammal in need of such treatment a therapeutically effective amount of the composition of Claim 36.
- 15 38. The composition of Claim 36 further comprising a therapeutically effective amount of an agent selected from the group consisting of a cytokine, colony stimulating factor, and interleukin.
- 20 39. The composition of Claim 38 wherein the agent is selected from KL, LIF, G-CSF, GM-CSF, M-CSF, EPO, IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 and IL-11.
- 25 40. A process for the biosynthesis of a human *mpl* ligand polypeptide, characterized by the amino acid sequence of one human *mpl* ligand polypeptide, such as that disclosed in Fig. 1 (SEQ ID NO: 1), comprising:
  - (a) growing a host cell culture containing a DNA isolate encoding the human *mpl* ligand polypeptide,
  - (b) recovering the human *mpl* ligand polypeptide from the culture, and
  - (c) purifying the *mpl* ligand polypeptide so as to obtain substantially homogeneous biologically active human *mpl* ligand polypeptide.

FIG. I A

FIG. I B

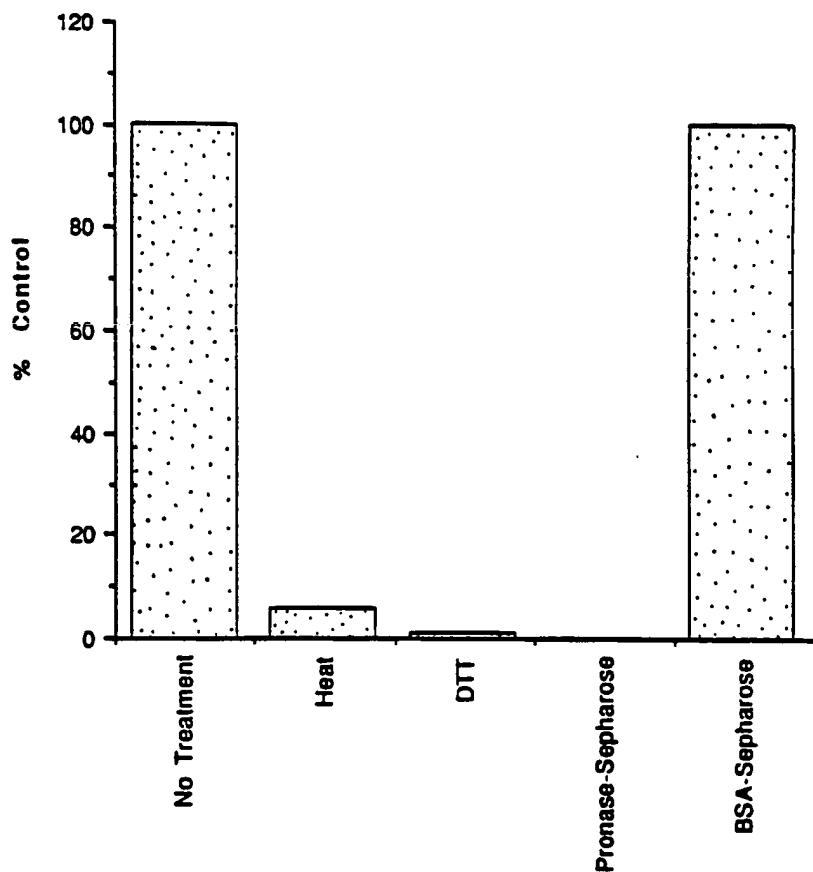


FIG.3

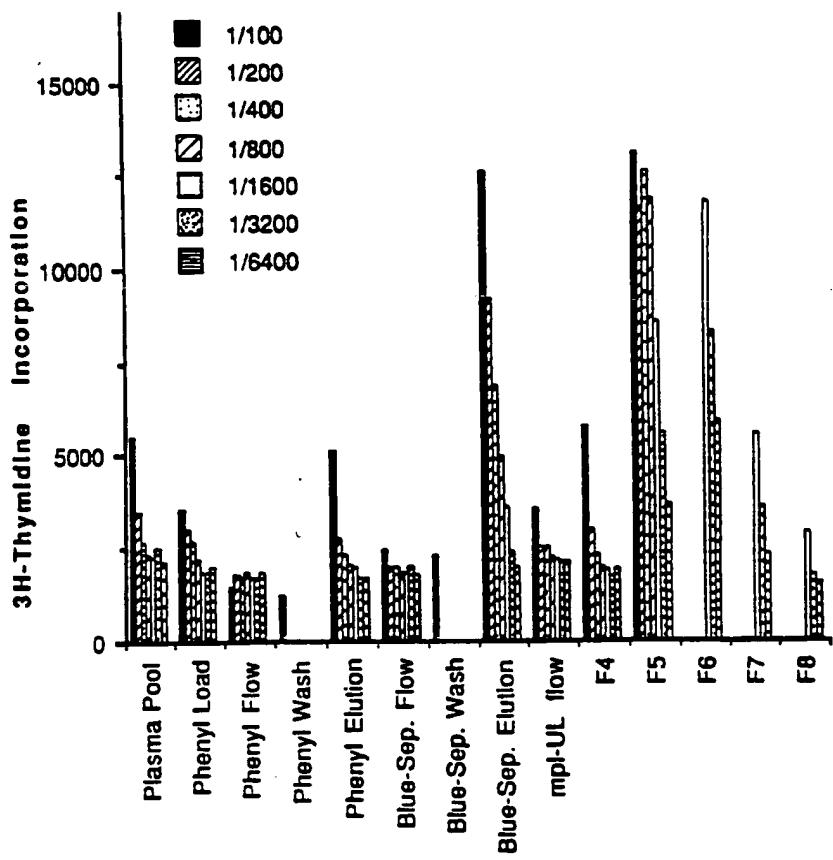


FIG.4

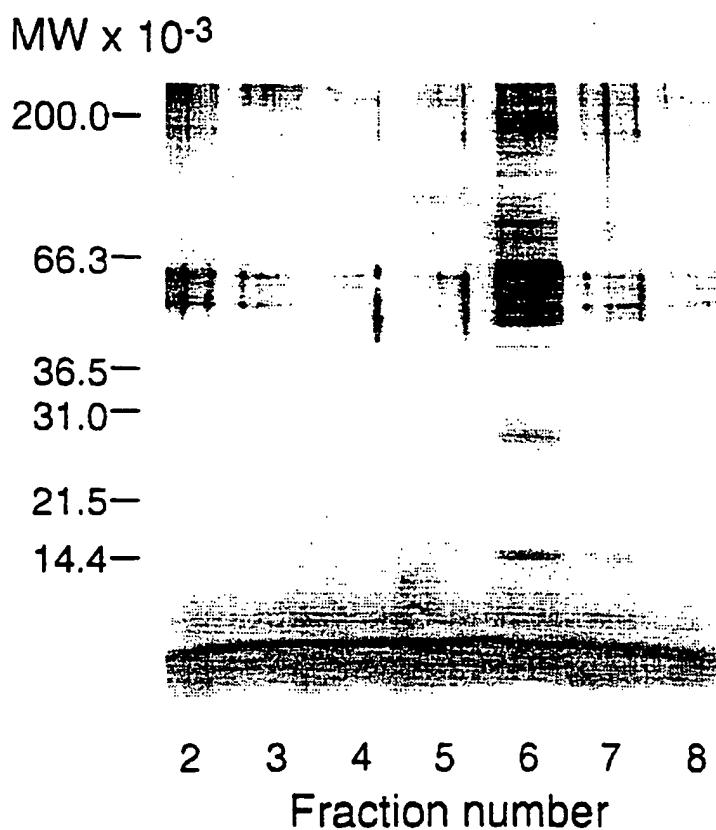


FIG. 5

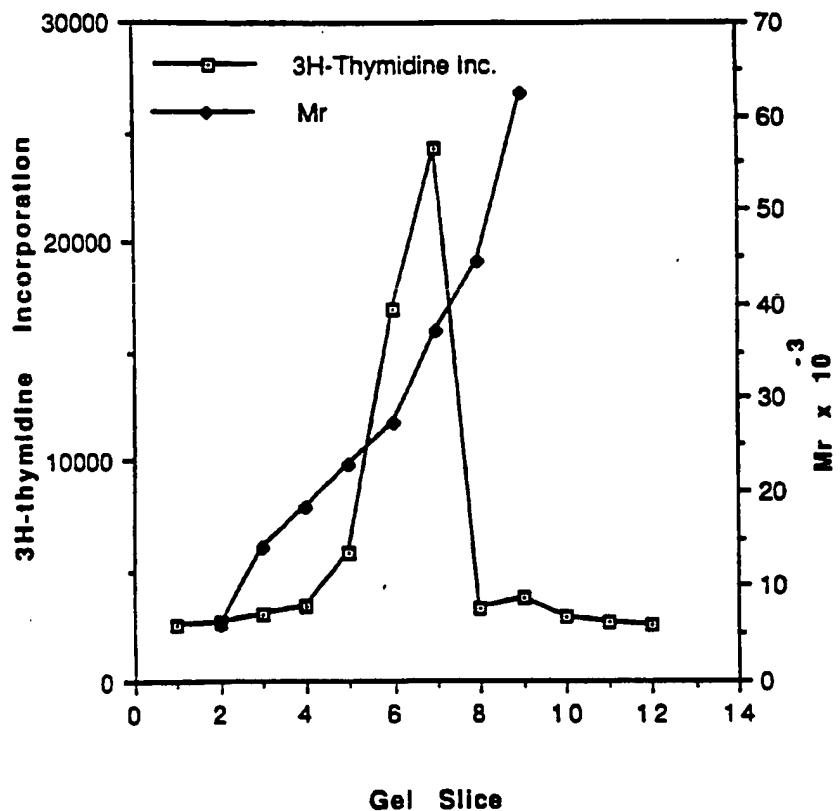


FIG.6

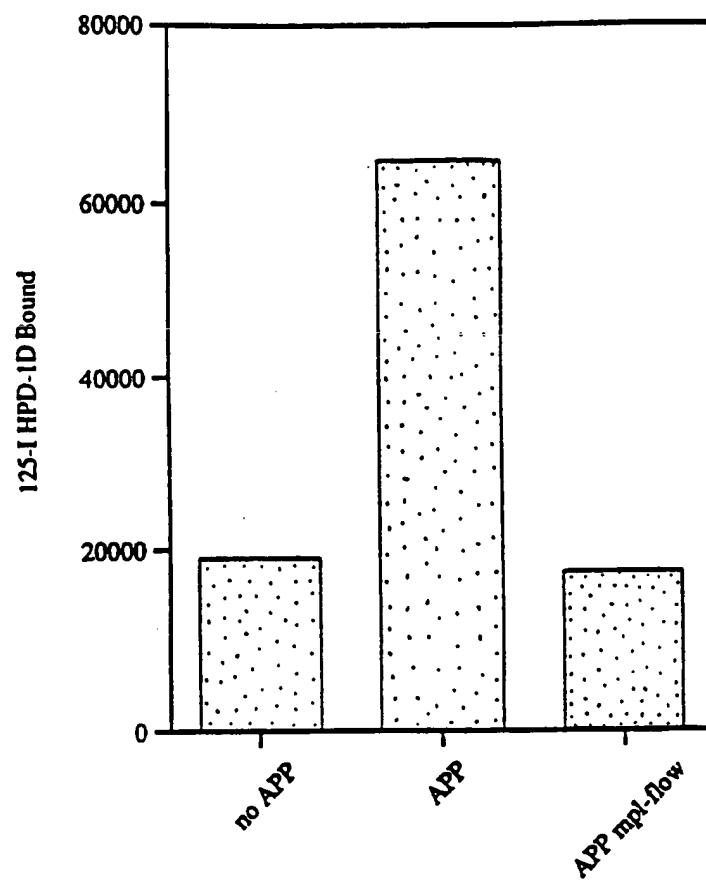


FIG. 7

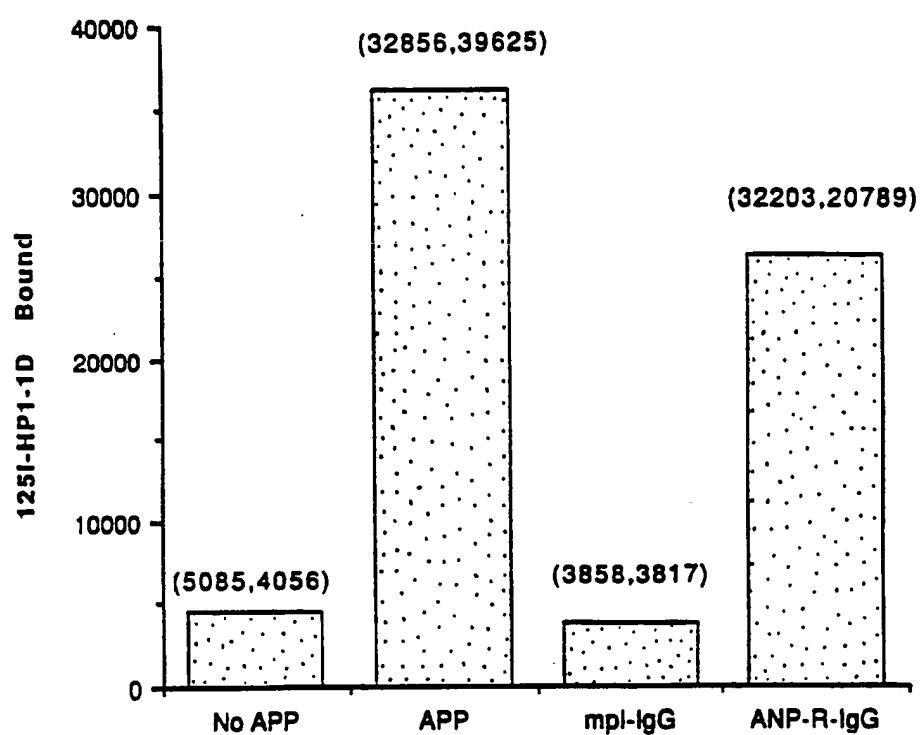


FIG.8

1 GATTCCCTGG AATACCAAGCT GACAATGATT TCCCTCCTCAT CTTTCACCT CACCTCTCTT CATCTAAGAA TTGCTCCTCG TGGCATGCTT TCTCCCTAACT  
 CTTAAGGACC TTATGGTGA CTGTTACTAA AGGAGGATA GAAGATGGA GTGGAGGGA GTAGATTCTT AAGGAGGAC ACCAGTAGCA AGAGGATTGA  
 10  
 A R L T L S S P A P P A C D L R V L S K L L R D S H V L H S R L  
 101 GCAAGGCTAA CGCTGTCAG CCCGGCTCCCT CCTCTGGAGT ACCTCCGAGT CCTCAGTAA CTGCTTCGGT ACTCCCATGT CCTTCACAGC AGACTCTGA  
 CGTTCGATT GCGACACCTC GGGCGAGGA GGACGACAC TGAGGCTCA GGAGTCATT GACGAAGCAC TGAGGTACA CGAAGTGTCA TCTGACCACT  
 20  
 201 GAACTCCCAA CATTATCCCC TTTATCCGGG TAATCTGGTA GACACCCATA CTCCCAGGA GACACCATCA CTTCCTCTAA CTCCCTGACC CAATGACTAT  
 CTTGAGGGTT GTAATAGGGC AAATAGGGC ATAGGACATT CTGTCGCTAT GAGGTCTT CTGTCGCTT GAGGGAGATT GAGGAACCTG GTTACTGATA  
 301 TCTTCACCA TTTGTCACAC CTACTGATCA CACTCTCTGA CAAGAATTAT TCCTCACAAAT ACAGCCCGCA TTAAAAGCT CTCGCTCTAGA  
 AGAGGGTAT AACAGGGGTG GATGACTGT GTGAGGACT GTGAGCTATA AGAGTGTAT TGTCTTAAAGT GAGGAGATCT  
 -10-

FIG.9

h-ML 1 S **P** **A** **P** **P** **A** **C** **D** **L** **R** **V** **L** **S** **K** **L** **R** **D** **S** **H** **V** **L** **S** **R** **L** **S** **Q** **C** **P** **E** **V** **H** **P** **L** **P** **T** **P** **V** **L** **P** **A** **V** **D** **F** **S** **L** **G** **E**  
h-epo 1 **A** **P** **P** **R** **L** **I** **C** **D** **S** **R** **V** **L** **E** **R** **V** **L** **E** **A** **K** **E** **A** **E** **N** **I** **T** **I** **G** **C** **A** **E** **H** **C** **S** **L** **N** **E** **N** **I** **T** **P** **D** **T** **K** **V** **N** **F** **Y** **A**

h-ML 51 **W** **K** **I** **Q** **M** **E** **E** **T** **K** **A** **Q** **D** **I** **L** **G** **A** **V** **I** **L** **L** **E** **G** **V** **M** **A** **R** **G** **Q** **G** **P** **T** **C** **L** **S** **-** **-** **S** **L** **G** **Q** **S** **G** **Q** **V** **R**  
h-epo 51 **W** **K** **R** **H** **E** **V** **G** **O** **O** **A** **E** **V** **W** **O** **G** **A** **L** **S** **E** **A** **V** **L** **R** **G** **A** **L** **V** **N** **S** **S** **O** **P** **W** **E** **P** **L** **Q** **L** **H** **V** **D** **K** **A** **V** **S**

h-ML 99 **L** **L** **-** **-** **L** **G** **A** **L** **O** **S** **L** **G** **I** **Q** **-** **-** **L** **P** **P** **O** **G** **R** **I** **I** **A** **H** **K** **D** **P** **N** **A** **I** **F** **L** **S** **F** **Q** **H** **L** **L** **R** **G** **K** **V** **R** **F** **L**  
h-epo 101 **G** **L** **R** **S** **L** **T** **T** **L** **R** **A** **L** **G** **A** **Q** **K** **E** **A** **I** **S** **P** **D** **A** **S** **A** **A** **P** **L** **R** **I** **I** **A** **D** **I** **F** **R** **K** **L** **F** **R** **V** **Y** **S** **N** **F** **L** **R**

h-ML 143 **-** **-** **M** **I** **V** **G** **G** **S** **T** **L** **C** **V** **R** **R** **A** **P** **P** **T** **A** **V** **P** **S** **R** **T** **S** **L** **V** **L** **T** **I** **N** **E** **L** **P** **N** **R** **T** **S** **G** **L** **E** **T** **N** **F** **A** **S**  
h-epo 151 **G** **K** **L** **K** **L** **T** **G** **E** **A** **C** **R** **T** **G** **D** **R**

h-ML 191 **R** **T** **T** **G** **S** **G** **L** **L** **K** **W** **Q** **G** **F** **R** **A** **K** **I** **P** **G** **L** **L** **N** **G** **T** **S** **R** **S** **L** **D** **Q** **I** **P** **G** **Y** **L** **N** **R** **I** **H** **E** **L** **L** **N** **G** **I** **R** **G** **L**  
h-epo

h-ML 241 **P** **G** **P** **S** **R** **R** **T** **L** **G** **A** **P** **D** **I** **S** **G** **I** **S** **D** **T** **G** **S** **L** **P** **P** **N** **L** **Q** **P** **G** **V** **S** **P** **T** **H** **P** **P** **T** **G** **Y** **T** **L** **F** **P** **L** **P** **P**

h-ML 291 **T** **L** **P** **T** **P** **V** **V** **Q** **L** **H** **P** **L** **L** **P** **D** **P** **S** **A** **P** **T** **P** **T** **P** **L** **N** **T** **S** **Y** **T** **H** **S** **S** **O** **N** **L** **S** **O** **E** **G**

FIG.10

FIG. II A

**FIG. I-B**

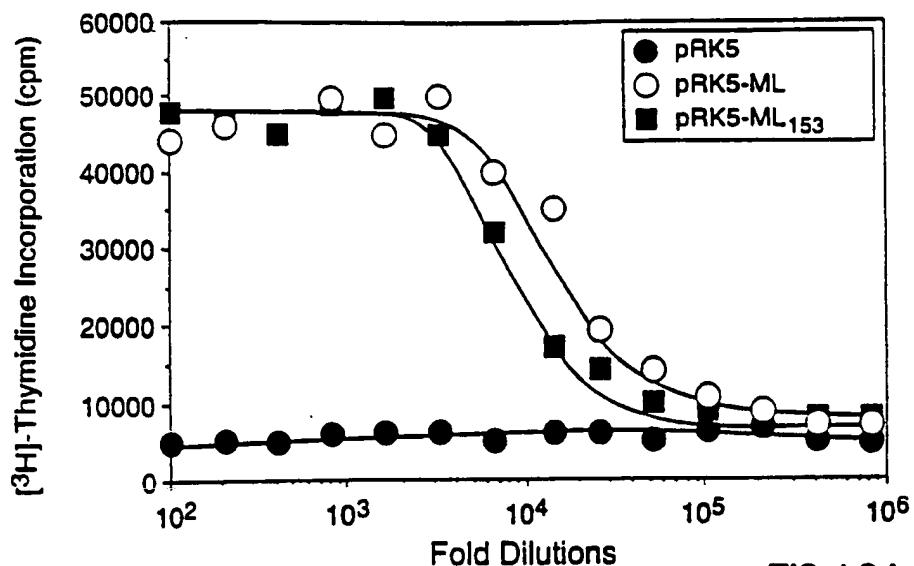


FIG. 12A

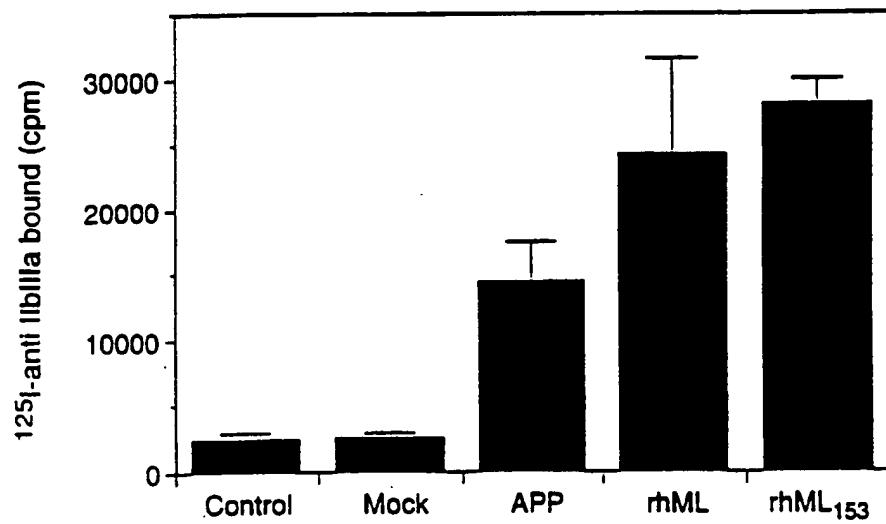


FIG. 12B

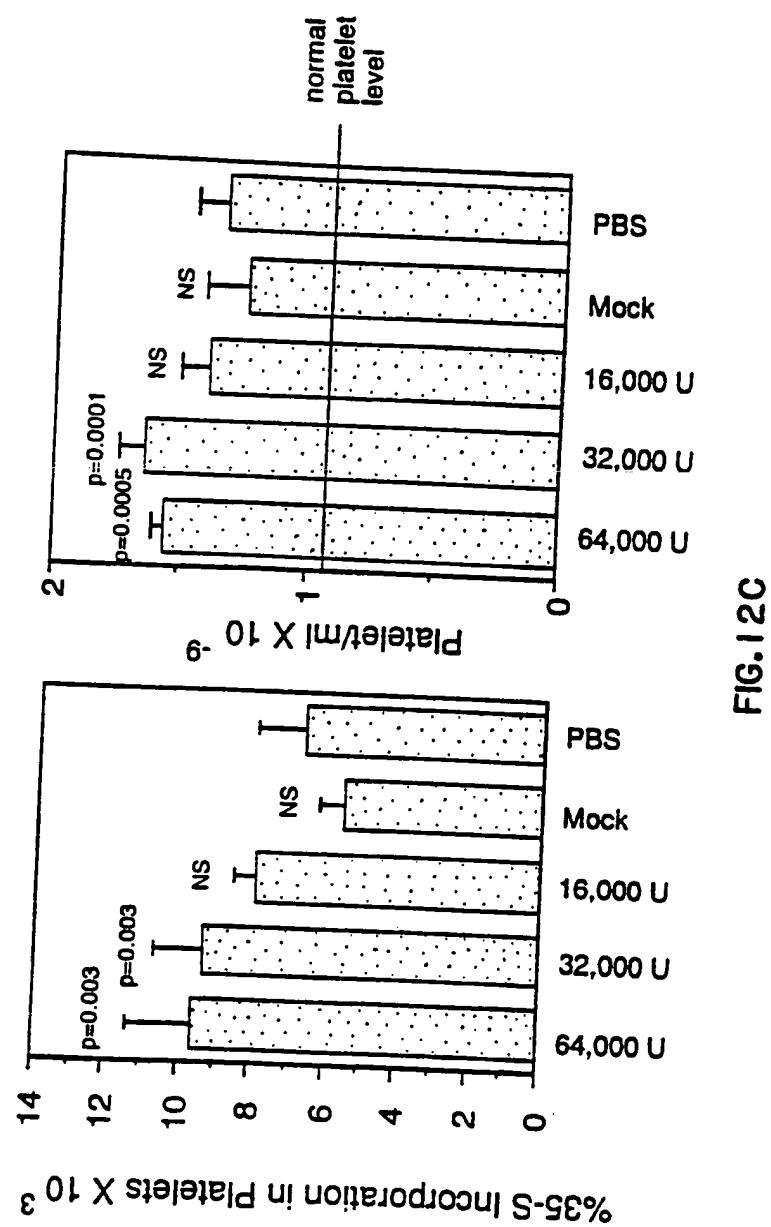


FIG. I 2C

FIG. I 4 A

3201 tcaggctctgggtctgaaatccctggatcacgctgacatgtttcaaacctcatcttaaggAAATTGCTCTC  
6 JulaLeuLeu FIG. I 4B

^ 2 05

Exon 2 < >

6

6

FIG. I-4B

9 ValValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSerHisVal  
 3301 GTGGTCATGCTTCTCTAACTGCTTAAGGCTTAAGGCTTAAGGCTCAGCCCCGGCTCCTCCCTGACTCCGAGCTCCGACTTCGTTGACTTCCCATG  
 End of signal peptide

FIG. 14C

FIG. 14D

FIG. I 4E

ML 332

---

ML 153

— 200

— 116  
— 97

— 66.3  
— 55.4

— 36.5  
— 31.1

— 21.5 —

FIG. 15

FIG. I 6A

170 AsnLysPhe ProAsnArgI hrSerGlyLe uLeuGluThr AsnPheSerV alThrAlaAr 9ThrAlaGly ProGlyLeuL euSerArgLe uGlnGlyPhe  
 701 AACAAAGTTCC GAAACAGGA CTTCTGGTT GTCGGAGACG AACCTCAGTG TCACAGCCAG CCTGGACTTC TGACCGCTC TCAGGGATTTC  
 801 AGACTCAAGA TTACTCTGG TCAGCTTAAAT CAAACCTCCA GTCAGCTTCA GTCAGCTTCA GTCAGCTTCA CGGACCTGTC ATGGAACTC  
 180 190 200  
 ArgValLysI lethr-ProG1 yGlnLeuL GlnThrSerA rgsSerProva 1GlnIleSer GlyTyxLeuA snargThrHi sGlyProva1 AsnGlyThrHis  
 210 220 230  
 GlyValLysI lethr-ProG1 yGlnLeuL GlnThrSerA rgsSerProva 1GlnIleSer GlyTyxLeuA snargThrHi sGlyProva1 AsnGlyThrHis  
 240 250 260  
 GlyLeuPh eAlaGlyThr SerLeuGlnT hrLeuGluAl aSerAspIa SerProGlyA 1aPheAsnL eGlySerLeu AlaPheAsnL euGlnGlyGly  
 901 ATGAGCTCTT TGCTGGAAAC TCACTTCAGA CCCTGGAAAGC CTCAGACATC TGCCCGGGAG CTTTCACAAAG AGGCTCCCTG GCATTCAACCG TCCAGGCTGG  
 270 280 290 300  
 LeuProPro SerProSerL euAlaProA pglyHiSthr ProPheProP roSerProA1 aLeuProThr ThrHiSglyS erProProG1 nLeuLisPro  
 1001 ACTTCCTCCT TCTCCAAGCC TTGGCTCTGA TGGACACACA CCCTTCCCTC CTTCACCTGC CTTGCCCCACCC ACCCTGAT CTACACCCCA GCTCCACCC  
 310 320 330  
 LeuPheProA sProSerL rThrMetPro AsnSerThrA 1aProHisP rThrMet TyrProHisP roValThrMet TyrProAsnLe uSerGlnGlu Thr  
 1101 CTGTTCCCTG ACCCTTCAC CACCATGCCCT AACCTTACCG CCCCTCATC AGTCACAAAG TACCCCTCATC CCACGGATTG GTCTCAGGAA ACTTAGGGCG  
 1201 GGCACCTGGCC CAGTGAGGGT CTGAGGCTC TCTGGGGAC AGGCTTCCCC AGGAGGCTG AGAGGAGCT GCATCTCTC CAGATCTCTT GCCTTCACCT  
 1301 AAAGGCCCT GGGGAAGGGA TACACAGCAC TGGAGATTGT AAATTTAG GAGCTATTG TTTTAACCT ATAGCAATA TTCAATCAGAG CAGCTAGGA  
 1401 TCTTTCGCTT ATTTTCGGTA TAATTTGAA ATCTACTAAT TCT

FIG. I 6B

FIG. 17A

1401 agcagcttagcgatctttggtctatttcggtataat  
1501aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

FIG. 18

hML3	1	S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E
mML3	1	S P V A P A C D P R L L N K L L R D S H L L H S R L S Q C P D V D P L S I P V L L P A V D F S L G E
hML3	51	W K T Q M E E T K A Q D I L G A V I L L E G V M A A R G Q L G P T C L S S L L G Q L S G Q V R L L
mML3	51	W K T Q M E E T Q S K A Q D I L G A V S L L E G V M A A R G Q L E P S C L S S L L G Q L S G Q V R L L
hML3	101	L G A L Q S I L G T Q L P P Q G R T T A H K D P N A I F L S F Q H L L R G K D F W I V G D K L H C I L
mML3	101	L G A L Q G L L G T Q L P L Q G R T T A H K D P N A L F L S L Q Q L L R G K D F W I V G D E L Q C H
hML3	151	S Q N Y W L W A S E V A A G I Q S Q D S W S A E P N L Q V P G P N P R I P E Q D T R T L E W N S W
mML3	151	S Q N C W P W T S E Q A S G I Q S Q D Y S W S A K S N L Q V P P S P N L W I P E Q D T R T C E W N S W
hML3	200	T L S W I T T A D P R S P G H F L R N I R H R L P A T Q P P A W I F S F P N P S S Y W T V V A L P S
mML3	201	A L C W N L T S D P G S I R H L A B S F Q Q R L P G I Q P P G W I T S S F S K P C S
hML3	250	S T H L A H P C G P A P P P A S

FIG. 18

m-ML	1	S P V A P A C D P R L L N K L L R D S H I L L H S R I L S Q C P D Y D P L S I P V L L P A V D F S L G E	
p-ML	1	S P A P A C D P R L L N K L L R D S H I V L H G R I L S Q C P D I N P L S T P V L L P A V D F I L G E	
h-ML	1	S P A P A C D L R V Y L S K L L R D S H I V L H S R I L S Q C P E Y H P I P V Y L L P A V D F S L G E	
•			
m-ML	51	W K I O T E Q S K A Q D I L G A Y S I L L E G Y M A A R G O L E P S C L S S I L G O L S G O V R I L L	
p-ML	51	W K I O T E Q S T K A Q D I L G A I T I L L E A V M I A R G O V G P P C L S S I L Y o l s G o v r i l l	
h-ML	51	W K I O T E M E E I K A Q D I L G A V I L L E G Y M A A R G O L G P C I C L S S I L G O L S G o v r i l l	
•			
m-ML	101	L G A L O G G L E G I O L P I O G R I T I A H K D P N A L F I S I O Q L L R G K V R F L L I V E G P T I L	
p-ML	101	L G A L Q D L L G H O L P O G R I T I A H K D P S A I F I N F O Q L L R G K V R F L L I V G P S I L	
h-ML	101	L G A L Q S L L G I O L P P O G R I T I A H K D P N A I F L S F d H L L R G K V R F l M L V G G S T L	
•			
m-ML	151	C V R R I L P I A V P S S I S Q L I L N K E P N R I S G I L E I S V I A R T A G P G L L I S R	
p-ML	151	C A K R A P P A I V P S S I S P F H I L N K L P S G L L E I S A R T I G S G F L K R	
h-ML	151	C V R R A P P T A V P S R I S L V I L N E P N R I S G I L E I S A R T I G S G L L K W	
•			
m-ML	201	L Q G F R Y K I I P G Q I I S R S P V O I S G Y L R I T H G P V N S I H G L F A G I S L Q F L E	
p-ML	201	L Q A F R A K I I P G L I I S R S L D O I P G H Q R E T H G P L S G I H G L F P G P O P G A L G	
h-ML	201	Q Q G F R A K I I P G L I I S R S L D O I P G Y L N R I H E L L N I G T R G L F P G P S R R R I L G	
•			
m-ML	251	A S D I S P G A F N K G S L A F N L Q G G L P P S P S I L A P D G H - T P F P P S P A L P I I H G S P	
p-ML	250	A P D I S P P A I T S G M G S R P T Y I O P G E S P S P A H P S P T S P S - - - P I	
h-ML	250	A P D I S S G I S D T G S L P P N L O P G Y S P S P I H P P I G O Y I L F P L P T I L P T - - - P V	
•			
m-ML	300	P Q L H P L F P D P S I I M P N I S T A P H P V I M Y P H P R I S Q E I	
p-ML	297	V Q L Q P L P D P S A I I T P N S S P L L F A A H P H F Q I S Q E E	
h-ML	297	V Q L H P L L P D P S A P I P T I S P L L F E Y I T H S O I S Q E G	

FIG. 19

FIG.20A

170 PheHisThrLeuAsnLysLeuProAsnArgThrSerGlyLeuLeuGluThrAsnSerSerAlaSerAlaArgThrThrGlySerGlyPheLeuLysArg  
 501 ATTCCACACTGAAACAGCTCCAAACAGGACCTCTGGATTTGGAGACAAACTCCAGTATCTCAGCCAGAACTACTGGCTCTGGATTCTCAAGAGG  
 180  
 190  
 200

210 LeuGlnAlaPheArgAlaLysIleProGlyLeuLeuAsnGlnThrSerArgSerGlySerGlnIleProGlyHisGlnAsnGlyThrHisGlyProLeuSer  
 601 CTGCAGGCATTCAAGCCAAGATTCTCGGTCAGAGCTCAGGCCAAACCTCCAGGATGACCAAAATCCCTGAGACACCAGAACGGACACACGGACCCTGAA  
 220  
 230

240 GlyIleHisGlyLeuPheProGlyProGlyAlaLeuGlyAlaProAspIleProProAlaThrSerGlyMetGlySerArgProThrTyrLeu  
 701 GTGGAATTCATGGACTCTTTCTGGACCCCAACCGGGCCCTCGGAGCTCCAGACATTCTCCAGCAACTTCAGGATGGCATGGCTCCGGCAACCTACCT  
 250  
 260

270 GlnProGlyGluSerProSerProAlaHisProSerProGlyArgTyrThrLeuPheSerProSerProThrSerProSerProThrValGlnLeuGln  
 801 CCAGGCCCTGGAGACTCTCTCCCTCCAGCTCACCCCTCAGCTCACCCACCTCCTTCACCCACCTCGCCCTCCCCACAGTCCAGCTCAG  
 280  
 290

300 ProLeuLeuProAspProSerAlaIleThrProAsnSerThrSerProLeuLeuPheAlaAlaHisProHisPheGlnAsnLeuSerGlnGluGlu  
 901 CCTCTGCTTCCTGACCCCTCTGGCGATCACACCCAACTACAGTCACCCCTCATTTGCAAGCTCAGGAAACTGTCAGGAAGAGTAAG  
 310  
 320  
 330

1001 GTGCTCAGACCCCTGCCCAACTTCAGCA

FIG.2OB

FIG. 2 | A

FIG. 2 | B

1001 TGCCTAACTTCAGCA

pML	1	SPAPPACDPALLNKLLRADSHVVLHGRILSQCPDINPLSTPVLLPAVDFTLGE
pML2	1	SPAPPACDPRLLNKLLRADSHVVLHGRILSQCPDINPLSTPVLLPAVDFTLGE
pML	51	WKTQTEQTKAQDVLGATTLLLEAVMTARGQVGGPPCCLSSLLVQLSGQVRL
pML2	51	WKTQTEQTKAQDVLGATTLLLEAVMTARGQVGGPPCCLSSLLVQLSGQVRL
pML	101	LGALQDLLGMQLPQGRTTAHKDPSAIFLNFQOLLRGKVRFLVVVGPSL
pML2	101	LGALQDLLGMQLPQGRTTAHKDPSAIFLNFQOLLRGKVRFLVVVGPSL
pML	151	CAKRAPPAlAVPSSSTSPFHTLNKLPNATSGILLETNSSISARTTGSGFLKR
pML2	147	CAKRAPPAlAVPSSSTSPFHTLNKLPNATSGILLETNSSISARTTGSGFLKR
pML	201	LQAFRAKIPGLLNOTSRSLDQ1PGHONGTHGPLSGIHGLFPGPQPGALGA
pML2	197	LQAFRAKIPGLLNOTSRSLDQ1PGHONGTHGPLSGIHGLFPGPQPGALGA
pML	251	PDIAPPATSGMGSSRPTYLQPGEESPSPAHPSPTSPSPPTIVQLQ
pML2	247	PDIAPPATSGMGSSRPTYLQPGEESPSPAHPSPTSPSPPTIVQLQ
pML	301	PLLPDPSSAITPNSTSPLLFAAHPHFAQNLSQEE
pML2	297	PLLPDPSSAITPNSTSPLLFAAHPHFAQNLSQEE

FIG.22

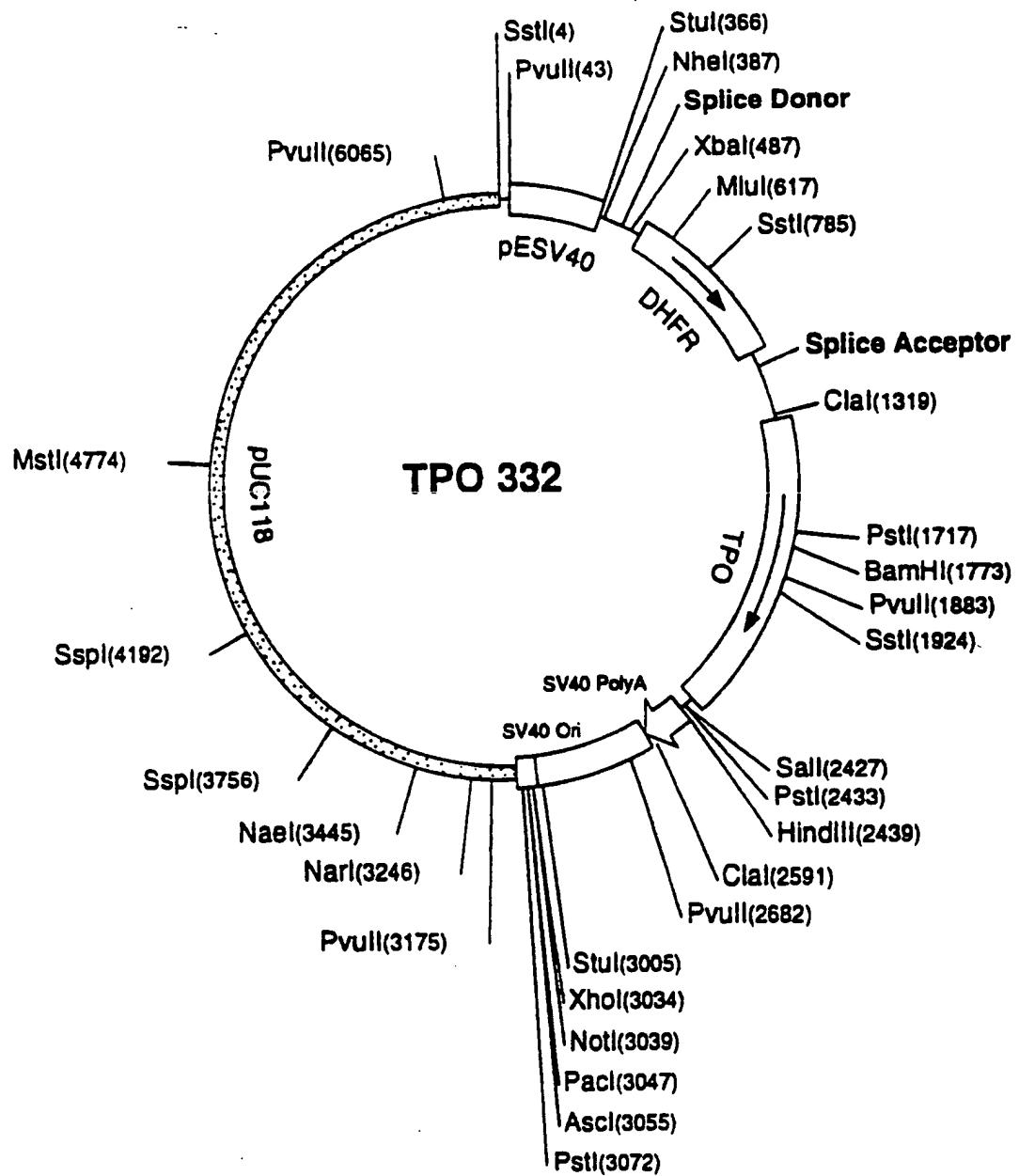


FIG.23

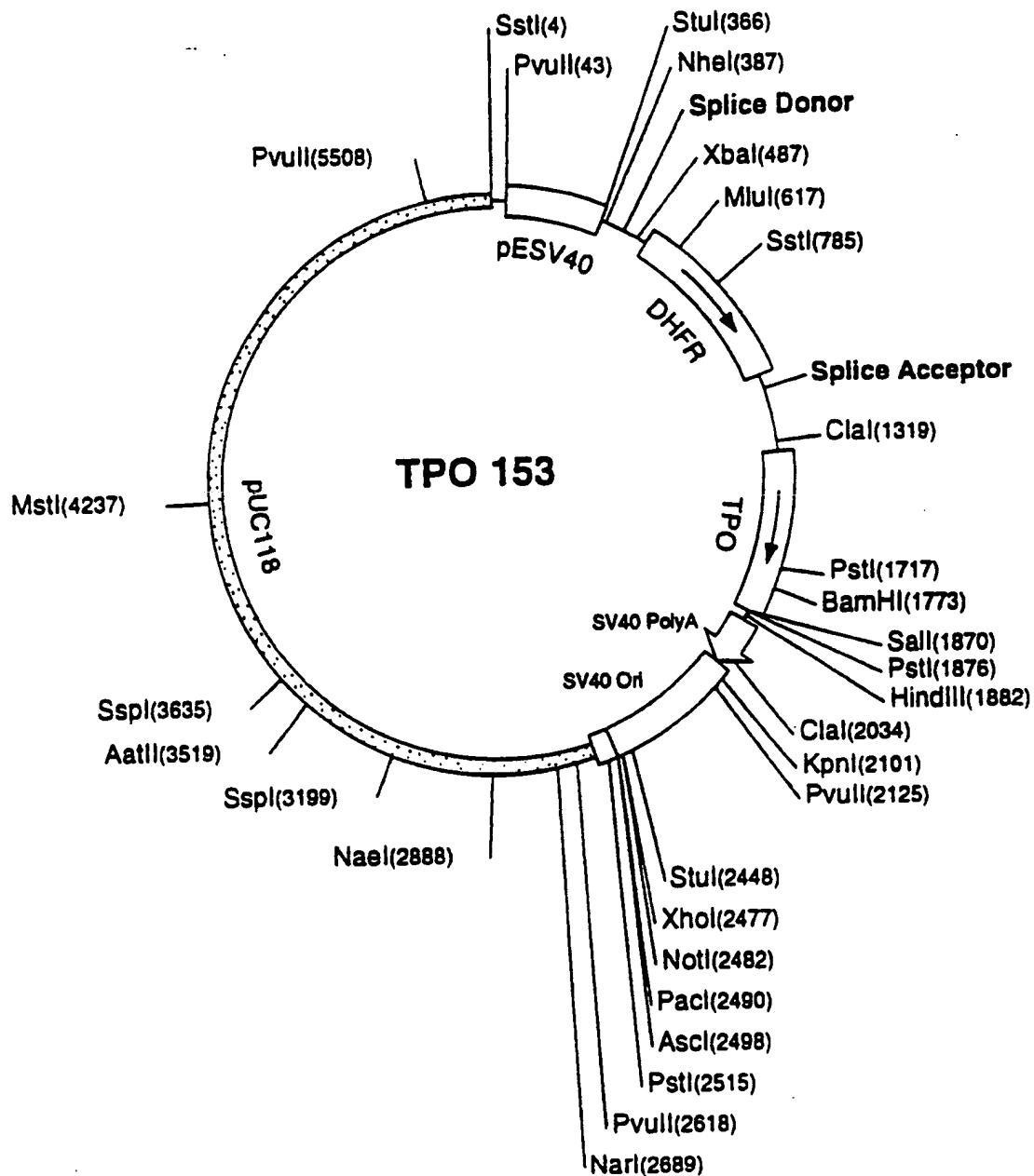


FIG.24

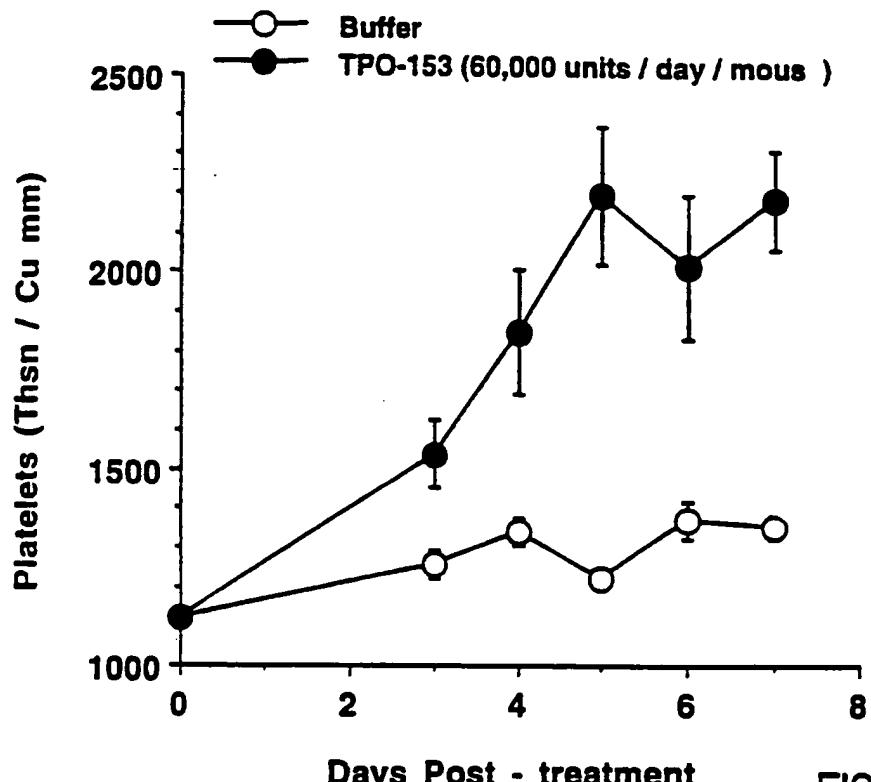


FIG.25A

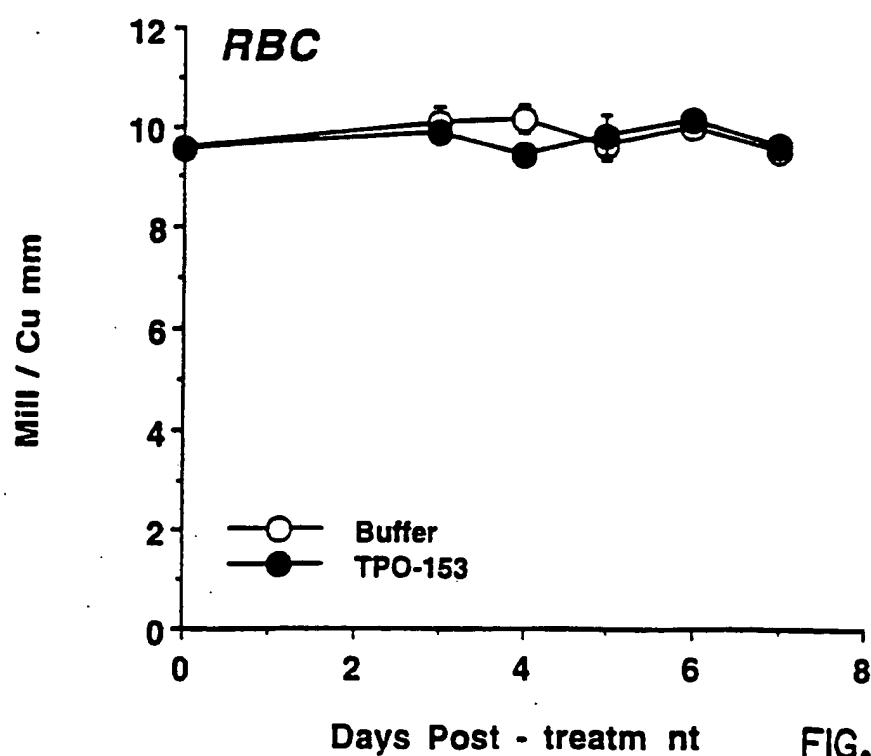


FIG.25B

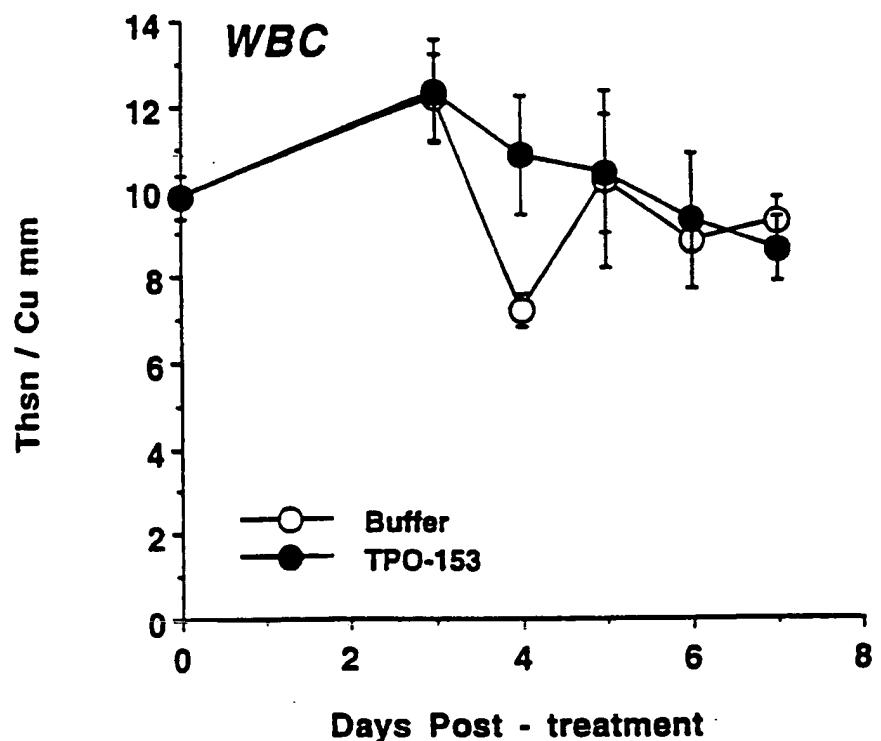


FIG.25C

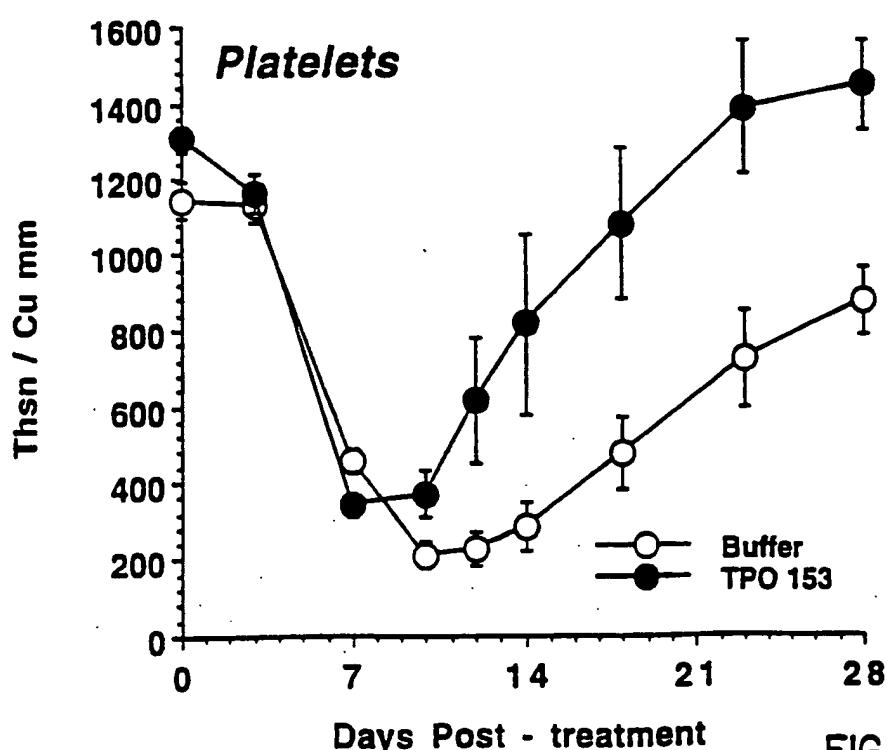


FIG.26A

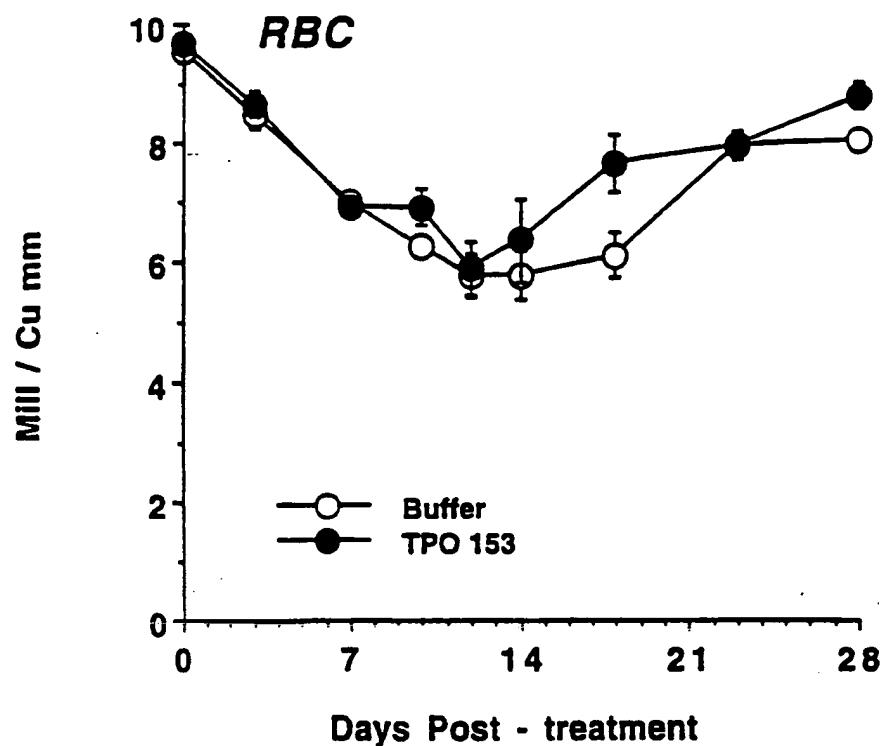


FIG.26B

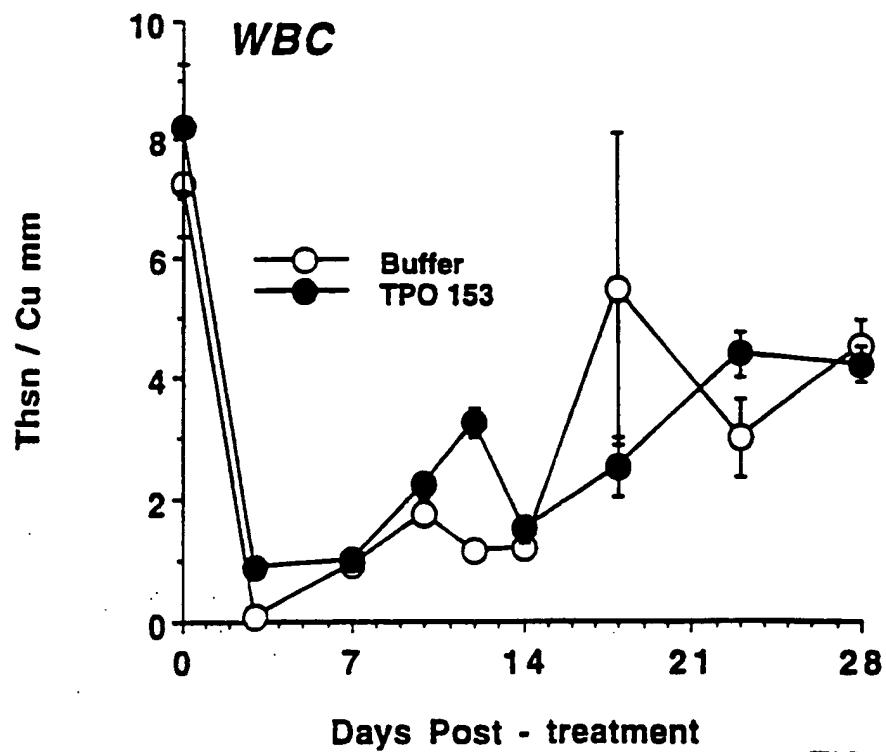


FIG.26C

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, vol. 106,no. 2, August 1985 pages 162-174, MCDONALD ET AL 'STUDIES ON THE PURIFICATION OF THROMBOPOIETIN FROM KIDNEY CELL CULTURE MEDIUM' see the whole document ---	1-5,12, 36-39
X	EXPERIMENTAL HEMATOLOGY, vol. 2,no. 6, 1974 pages 355-361, MCDONALD ET AL 'PURIFICATION AND ASSAY OF THROMBOPOIETIN' see the whole document ---	1-5,12, 36-39
A	THE EMBO JOURNAL, vol. 12,no. 7, 1993 pages 2645-2653, SKODA ET AL 'MURINE C-MPL:A MEMBER OF THE HEMATOPOIETIC GROWTH FACTOR RECEPTOR SUPERFAMILY THAT TRANSDUCES A PROLIFERATIVE SIGNAL' cited in the application ---	
P,X	NATURE, vol. 369, 16 June 1994 pages 533-538, DE SAUVAGE ET AL 'STIMULATION OF MEGAKARYOCYTOPOIESIS AND THROMBOPOIESIS BY THE C-MPL LIGAND' see the whole document ---	1-40
P,X	CELL, vol. 77, 1 July 1994 pages 1117-1124, BARTLEY ET AL 'IDENTIFICATION AND CLONING OF A MEGAKARYOCYTE GROWTH AND DEVELOPMENT FACTOR THAT IS A LIGAND FOR THE CYTOKINE RECEPTOR MPL' see the whole document ---	1-40
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 91,no. 26, 20 December 1994 pages 13023-13027, FOSTER ET AL 'HUMAN THROMBOPOIETIN:GENE STRUCTURE,CDNA SEQUENCE,EXPRESSION,AND CHROMOSOMAL LOCALIZATION' see the whole document ---	1-40
1		-/-

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>FEBS LETTERS, vol. 353, no. 1, 10 October 1994 pages 57-61, SOHMA ET AL 'MOLECULAR CLONING AND CHROMOSOMAL LOCALIZATION OF THE HUMAN THROMBOPOIETIN GENE' see the whole document</p> <p>-----</p>	1-40